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METABOLIC FRACTIONATION OF C^{13} & C^{12} IN PLANTS [1, 2]

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Nier and Gulbransen (10) observed 20 years ago that the C^{13}/C^{12} ratio of carbon in nature ($\sim 1/90$) varies slightly depending on the source of the carbon analysed. These workers found that limestones, atmospheric carbon dioxide, marine plants, and land plants possess different and characteristic C^{13}/C^{12} ratios. Workers using improved analytical techniques (9) have confirmed and extended many of Nier and Gulbransen's early observations (4, 5, 7, 13, 14, 15). Our present knowledge of the C^{13}/C^{12} ratio ranges for various natural carbon containing materials is summarized in figure 1. The results in figure 1 are reported in terms of a δ value. The δ value indicates the difference in per mil of the C^{13}/C^{12} ratio of the sample relative to a standard and is defined as:

$$\delta \text{ in per mil } (‰) = \frac{C^{13}/C^{12} \text{ sample} - C^{13}/C^{12} \text{ standard}}{C^{13}/C^{12} \text{ standard}} \times 10^3 .$$

In this work the standard is CO_2 prepared from the fossil skeleton of a Cretaceous belemnite, *Belemnitella americana*, from the Peedee formation of South Carolina. This scale is called the PDB scale (6). A δ value of + 10 means that the C^{13}/C^{12} ratio of the sample is greater than that of the standard by 10 per mil or 1 %. Conversely a δ value of −10 means that the C^{13}/C^{12} ratio of the sample is less than that of the standard by 10 per mil or 1 %. The precision of the data presented in figure 1 is about ±0.01 per cent or ±0.1 per mil of the ratio (9).

The data of figure 1 show that many steps in the carbon cycle are accompanied by isotope fractionation. One of the most important processes in nature which causes fractionation of carbon is photosynthetic carbon assimilation by green plants. Both terrestrial and marine plants have lower C^{13}/C^{12} ratios than their respective carbon sources, atmospheric CO_2 and ocean carbonates. This means that during photosynthetic CO_2 fixation there is preferential utilization of C^{12} and exclusion of C^{13}.

Investigations on the magnitude and mechanism of the C^{13}/C^{12} fractionation which occurs during photosynthesis have been made by Baertschi (1, 2) and by Park and Epstein (12). These workers observed that plants grown in carbon dioxide of controlled C^{13}/C^{12} ratio discriminated against C^{13} during photosynthetic CO_2 fixation. The fixed plant carbon was −26 per mil with respect to the atmospheric CO_2. Park and Epstein proposed and experimentally supported a model for photosynthetic fractionation of carbon which consisted of two stages. The first fractionation stage in favor of C^{12} was a kinetic effect which occurred during uptake of CO_2 from the atmosphere into the leaf cytoplasm. In rapidly photosynthesizing tomato plants, carbon dioxide dissolved in the cytoplasm was

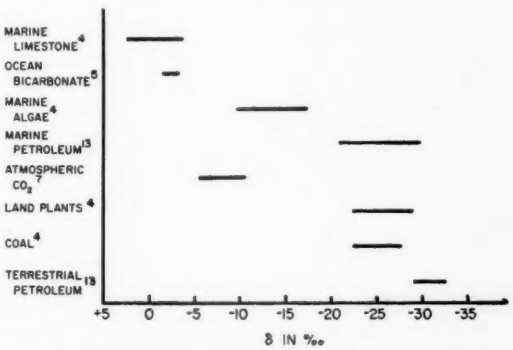

FIG. 1. Range of C^{13}/C^{12} ratios for various carbon reservoirs in nature.

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enriched in C^{12} compared to atmospheric CO_2 by 7 per mil. The second fractionation stage in favor of C^{12} occurred during fixation of dissolved CO_2 via the carboxydismutase enzyme into 3-phosphoglyceric acid (PGA). The experimentally observed fractionation between bicarbonate and carbon fixed into PGA by the isolated enzyme was 15 per mil. The sum of these two separate fractionations is about equal to the total fractionation observed during photosynthesis of experimentally grown tomato plants. These observations were used not only to explain C^{13}/C^{12} fractionation during photosynthesis, but also to explain the variations in C^{13}/C^{12} ratio of plants in nature.

The data in figure 1 have many other interesting features. For example, coals of all geological ages have about the same C^{13}/C^{12} ratios as present day plants. Appparently coal represents organic plant residue which is not fractionated during coal formation. On the other hand, petroleums of marine and terrestrial origin are both enriched in C^{12} with respect to their present day plant sources. Since coals and limestone of all geologic ages have about the same C^{13}/C^{12} ratio, the C^{13}/C^{12} ratio of atmospheric carbon dioxide has probably also been constant over geologic time. For these reasons, C^{12} enrichment in petroleum cannot be explained by geologic periods in which plant sources for petroleum were unusually enriched in C^{12}. A possible explanation for the C^{12} enrichment in petroleum hydrocarbons is that plants produce a C^{12} enriched fraction which is selectively preserved during sedimentation and petroleum formation while the remainder of the plant is oxidized. Metabolic fractionation of carbon compounds following photosynthetic carbon fixation would be necessary to produce a C^{12} enriched petroleum precursor. If measurable metabolic fractionation of carbon is occurring in plants, it should be possible to find chemical materials within the plant which differ in $C^{13}/^{12}$ ratio. This paper reports the results of a search for a C^{12} enriched plant fraction which might give rise to petroleum hydrocarbons.

Materials and Methods

Preparation of Lipid Fraction: The whole dried plant material was ground up with a mortar and pestle. The plant material was then extracted with ethanol in order to allow more complete subsequent extraction with chloroform. The ethanol extract was also extracted with chloroform and the two cholorform extracts were pooled. The following evidence indicates that no isotopic exchange between the solvent and solute took place during the extraction procedure.

Two samples, each containing 1 gm of dried Ulva sp. were extracted at room temperature with 10 cc of ethanol and 10 cc of chloroform, respectively. The extract was filtered after 6 hours and was transferred to a refluxing apparatus. At the time intervals shown in table I, 2 cc of solvent were removed from the reaction flask. After evaporation of the solvent, the residue was converted to CO_2 for analysis. During the interval 24 to 30 hours, the samples were refluxed. The consistency of the C^{13}/C^{12} ratios of the solutes in table 1 indicate that no measurable exchange of carbon between solvent and solute took place during this procedure.

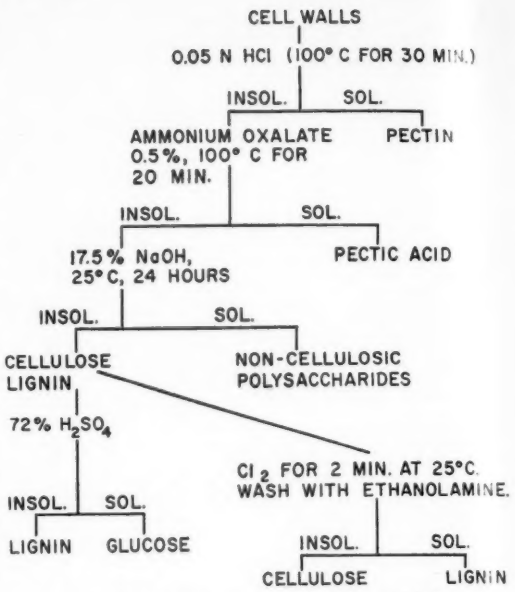

Fig. 2. Fractionation of cell wall material.

Fractionation of Cell Wall Material: The dried cell walls obtained as residue in the preparation of the lipid fraction were fractionated according to the scheme in figure 2. This method for plant cell wall fractionation is described by Ordin, Cleland, and Bonner (11) with the exception of the lignin and cellulose preparations which were prepared according to the methods cited by Bonner (3). The HCl and NaOH fractions were reduced to small volume in a desiccator and precipitated with 95 % ethanol.

Collection of Respired CO_2: Tomato plants (*Lycopersicon esculentum* Mill.) grown from CO_2 of known isotopic composition were used for the collec-

Table I

Exchange of Carbon Isotopes Between Solvent & Plant Lipid

Elapsed time, hr	δ of Ethanol soluble material, ‰	δ of Chloroform soluble material, ‰
6	−21.4	−22.5
24	−21.3	−22.5
30	−21.4	−22.4

tion of respired CO_2. The apparatus used for this study consisted of a respiratory chamber, about one liter in volume, followed by a dry ice trap and two liquid nitrogen traps in series. The plants were placed in a darkened respiratory chamber which was then flushed with 20 volumes of CO_2 free air prepared by washing with Ascarite. No CO_2 could be detected in air washed in this way either by an infrared analyzer, $Ba(OH)_2$ absorption, or absorption in a liquid nitrogen trap. Stopcocks were adjusted so that 70 cc of CO_2 free air swept through the system each minute. The respired CO_2 was then collected at desired time intervals. The respiratory chamber and dry ice trap were maintained at atmospheric pressure while the liquid nitrogen traps were at 1 cm mercury pressure. The respired CO_2 was completely removed by the first liquid nitrogen trap. Except for samples collected shortly after initiation of the experiment, samples were collected over a period of one hour. The collected CO_2 was transferred to the second trap and then to the mass spectrometer sample tube. The sample volumes were measured manometrically.

Combustion of Samples to CO_2: Plant materials were converted to CO_2 according to the procedure described by Craig (4). In this procedure samples are combusted over copper oxide in an oxygen atmosphere at 800 to 900° C. After cycling the gases through the combustion tube with an automatic Toepler pump for 30 minutes, the CO_2 produced is collected in a liquid nitrogen trap. The trap is then warmed in a dry ice bath and the CO_2 distilled into another trap at liquid nitrogen temperature. After several such transfers the CO_2 is free of water and most other contaminants. The CO_2 is then distilled into a sample tube and analyzed.

Mass Spectrometer Analysis: The CO_2 samples were analyzed in a Nier 60 ° sector type mass spectrometer which incorporates the improvements described by McKinney, et al for determining C^{13}/C^{12} ratios to ± 0.1 per mil (9). All samples are corrected for the O^{17} contribution to the mass 45 peak and for mixing of the sample and standard gas (6). Each of the δ values presented in the Results section represents an individual sample.

Results

C^{13}/C^{12} Ratios of Various Plant Fractions: In two experiments tomato plants were grown in air containing 1.5 % CO_2 by volume of known C^{13}/C^{12} ratio (12). A portion of the plants was separated into chemical fractions according to the scheme described above. These fractions were subjected to isotopic analysis (table II). The only fractions which differed greatly in C^{13}/C^{12} ratio from the plant as a whole were the chloroform soluble or non-polar lipid fractions. These fractions were of lower C^{13}/C^{12} ratio than the plant as a whole.

To establish the generality of C^{12} enrichment in the lipid fraction of various plant taxonomic groups, a number of other plant lipids were analyzed and compared to the isotopic composition of the plant as a whole. (table III). In all plants investigated, the C^{13}/C^{12} ratio of the lipid fraction was smaller than that of the whole plant.

Respired CO_2 Collected Over Long Intervals: The respired CO_2 from 19 thirteen-day-old tomato plants ($\delta = 25.2$ per mil) was collected at various intervals during a three week dark period. After the first 14 hours, 14 plants were removed for isotopic analysis. Five plants were left in the respiratory chamber and respired CO_2 was collected and analyzed

Table II

C^{13}/C^{12} Ratios of Chemical Fractions Within Tomato Plants Grown in 1.5 % CO_2

Fraction	δ ‰ for AP series	δ ‰ for BP series
Cellulose	−23.6	−25.1
Lignin	−24.2	...
Non-cellulosic polysaccharides	−23.1	...
Pectin	...	−23.6
Ethanol soluble, $CHCl_3$ insoluble	−25.9	−25.1
$CHCl_3$ soluble	−30.2	−29.6

Table III

C^{13}/C^{12} Ratios of Plants & Their Respective Lipid Fractions as Found in Nature

Plant	δ of Carbon, ‰ Whole plant	Lipid	Residue	Amt. chloroform soluble material, % dry wt
Ulva sp.	−15.7	−23.5	−15.4	4.0
Gelidium sp.	−20.5	−24.3	−19.9	9.4
Macrocystis pyrifera (L.) Ag.	−12.6	−21.0	−12.7	1.7
Mixed Phyto plankton*	− 9.2	−14.2	− 8.3	6.8
Asplenium bulbiferum Forst.	−29.2	−37.9	−30.7	3.8
Triticum vulgare Vill.	−28.5	−31.1	−27.6	11.6
Sequoia gigantea (Lindl.) Dec.	−26.4	−28.3	−25.2	6.4
Solanum tuberosum L.	−25.6	−33.6	−25.6	0.2

* Kindly supplied by Dr. William Thomas of the Scripps Institution of Oceanography. Relative cell counts on the sample were as follows: *Prorocentrum micans* Ehrenb. 798, *Gymnodinium* sp. 13 with several other forms present in very minor amounts.

TABLE IV

C^{13}/C^{12} RATIO OF CO_2 RESPIRED FROM TOMATO PLANTS ($\delta = -25.2‰$) OVER LONG TIME INTERVALS

NO. OF PLANTS	ELAPSED TIME FROM START OF EXP., hr	μmoles CO_2 RESPIRED BETWEEN COLLECTIONS	δ OF RESPIRED CO_2, ‰
19	14	1,230	−22.1
5	38	378	−23.9
5	86	462	−23.5
5	254	1,550	−25.7
5	518	1,880	−28.2

at the time intervals indicated in table IV. These plants ultimately began to decay. The CO_2 obtained near the end of the experiment was probably derived from bacterial decomposition of the plants. At the end of the experiment, the decomposed plant residue was collected and its isotopic composition measured. A material balance calculation for the respired CO_2 and residue as contrasted to the C^{13}/C^{12} ratio of the dried whole plants is made in table V. It may be concluded that there is good agreement between the isotopic composition of the plant as a whole and the value calculated from the respired CO_2 and the respiration residue.

TABLE V

MATERIAL BALANCE FOR RESPIRED CO_2 & RESIDUE AS COMPARED WITH INITIAL PLANTS

	MATERIAL (CALCULATED ON BASIS OF 1 PLANT)	AMOUNT OF C IN mmole	δ OF CO_2, ‰
1.	Intact plant (42.1 mg). Assume C = 43.6 % dry wt (8)	1.53	−25.2
2.	Respired CO_2	0.93	−26.2
3.	Residue. Assume C = 43.5 % dry wt (8)	0.73	−24.7
4.	Sum of lines 2 & 3	1.66	−25.6

RESPIRED CO_2 COLLECTED OVER SHORT INTERVALS: An experiment was done in which respired CO_2 was collected over much shorter time intervals than those in the first respiration experiment. Three 25-day-old, air grown tomato plants were placed in the respiratory chamber. The chamber was darkened and rapidly swept with CO_2-free air for 4 minutes. Carbon dioxide was then collected and analyzed at the intervals given in table VI. The rapid change in the δ values for the respired CO_2 over the first 10 minutes of the experiment probably represents the transition of dissolved CO_2 in the leaf from the photosynthetic state to the isotopically lighter respiratory state.

DISCUSSION

The results presented above show that the sugar polymers of the plant cell wall possess C^{13}/C^{12} ratios similar to the plant as a whole. This is not surprising, since cell wall material constitutes the bulk of the plant carbon. Lipids, on the other hand, are enriched in C^{12} compared to the whole plant, by as much as 8 per mil. The data in figure 3 show that the C^{13}/C^{12} ratio of plant lipids corresponds roughly to petroleum C^{13}/C^{12} ratios. The matching is especially good for the terrestrial petroleums. Contributions of terrestrial plant material to the marine environment may account for the somewhat low C^{13}/C^{12} ratio of marine petroleums. The fractionation, which enriched petroleums in C^{12} as compared to present day plants, may not have occurred by bacterial fractionation of plant cell wall carbon during sedimentation. Rather, the fractionation could have occurred within the living plant during the normal course of its fat synthesis. The difference in C^{13}/C^{12} ratio between present day plants and petroleums could result from the selective preservation of the C^{12} enriched plant lipid material during the decay of the plant organic matter. On the other hand, coal, which has about the same range of C^{13}/C^{12} ratios as present day plants, appears to represent preservation of representative plant organic material without preferential decomposition of either the lipid or carbohydrate fractions (see fig 1).

Any enrichment of C^{12} in one chemical component of the plant such as lipid must necessarily be accompanied by enrichment of C^{13} in some other component. The data of tables IV and VI show that respired CO_2 has a C^{13}/C^{12} ratio greater than the plant as a whole and is possibly the C^{13} enriched component which results from lipid formation. This proposal is supported by both biochemical and isotopic evidence. Respiration and lipid formation are closely related biochemical systems. The same two carbon fragment, acetate, is used both for lipid synthesis and for respiration in the tricarboxylic acid cycle. Isotope selection at the level of the two carbon unit acetate or the three carbon unit pyruvate provides an explanation for

TABLE VI

C^{13}/C^{12} RATIO OF CO_2 RESPIRED FROM TOMATO PLANTS ($\delta = 32.4$/mil) OVER SHORT TIME INTERVALS

SAMPLE NO.	ELAPSED TIME FROM START OF EXP.	μmoles CO_2 RESPIRED BETWEEN COLLECTIONS	δ, ‰
1	10 min	51	−24.3
2	2 hr	488	−25.8
3	4 hr	373	−27.2
4	6 hr	174	−27.6
5	16 hr	1,100	−28.2
6	40 hr	2,120	−30.0

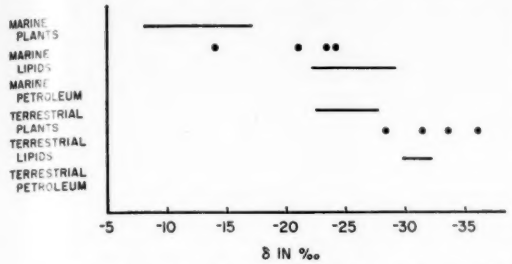

FIG. 3. C^{13}/C^{12} ratios of terrestrial and marine plants and petroleums as compared to the lipid fractions of terrestrial and marine plants.

the data obtained so far. Figure 4 outlines the biochemical pathways leading to lipid and respiratory CO_2 formation. That isotope selection actually does

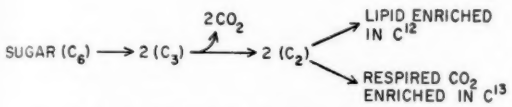

FIG. 4. The biochemical pathway leading from sugars to two carbon units and their subsequent metabolism.

occur at this point is indicated in figure 5, in which the degree by which the plant lipid is enriched in C^{12} above the level characteristic of the plant as a whole is plotted against the lipid content of the plant. It is noted that lipids are more enriched in C^{12} in those plants in which lipid is present in small amounts. This is consistent with the suggestion that C^{12} enriched C_2 units are selected for lipid synthesis. At the present time, the mechanism of this selection is a matter of speculation. It is interesting to note the wide taxonomic variety of plants which obey this relation.

Baertschi (1) observed that CO_2 respired by germinating beans possessed the same C^{13}/C^{12} ratio as the whole seed. Our data indicate that CO_2 respired by tomato plants is initially of larger C^{13}/C^{12} ratio than the plant as a whole. These two results are not difficult to reconcile on the basis of the biochemical selection process proposed above. In the case of seeds germinating in the dark, lipids are not being formed, but are being oxidized along with the carbohydrates as an energy source. The C^{13}/C^{12} ratio of the respired CO_2 should be that of the sugars and lipid being respired as a whole. However, if C^{12} enriched lipid is being synthesized rather than oxidized, as in the case of growing tomato plants, the respired CO_2 would be expected to have a larger C^{13}/C^{12} ratio than the whole plant. The data of table IV show that CO_2 of higher C^{13}/C^{12} ratio than the plant as a whole is respired by tomato plants only during the initial portions of the experiment. This period can be interpreted as the time during which lipid synthesis is still occurring. When lipid is no longer being produced, the respired CO_2 would have a C^{13}/C^{12} ratio similar to that of the plant as a whole. CO_2 of lower C^{13}/C^{12} ratio than the plant, evolved during the decomposition of the plant, may result from preferential decomposition of the lipids, fractionation during bacterial respiration, or a combination of these two phenomena.

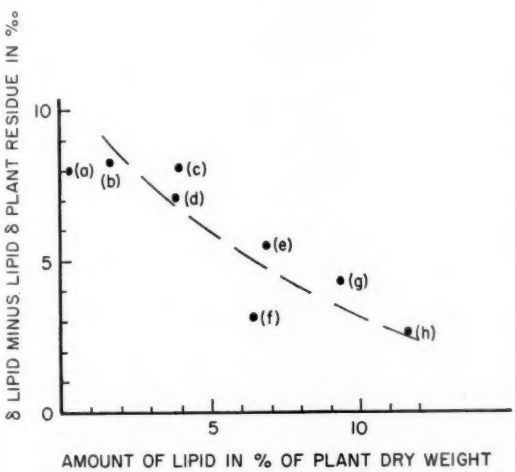

FIG. 5. C^{12} enrichment in lipid fraction as compared to the plant residue as a function of the amount of lipid in per cent of plant dry weight. (a) Potato, (b) brown alga, (c) green alga, (d) fern, (e) phytoplankton, (f) conifer, (g) red alga, (h) grass.

SUMMARY

C^{13}/C^{12} ratio analyses of chemical fractions from several plant phyla show that in all cases the lipid fraction is enriched in C^{12} compared to the whole plant. The C^{13}/C^{12} ratio of the plant lipids corresponds roughly to the C^{13}/C^{12} ratio of petroleums. The C^{12} enrichment in petroleums as compared to present day plants can be explained if selective preservation of plant lipids occurred during the sedimentation process. The degree of C^{12} enrichment in the plant lipid fraction is inversely related to the amount of lipid in the plant. The C^{12} enrichment which occurs in plant lipids may be balanced by the C^{13} enrichment which occurs in respired CO_2. Isotope selection at the level of acetate or pyruvate is a possible mechanism for explaining our results.

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ANALYTICAL STUDY OF IPOMEAMARONE & CHLOROGENIC ACID ALTERATIONS IN SWEET POTATO ROOTS INFECTED BY CERATOCYSTIS FIMBRIATA[1, 2]

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Several metabolic alterations have been studied, from biochemical and pathological viewpoints, in sweet potato roots infected by the black rot fungus, *Ceratocystis fimbriata* (2, 11, 13), among which the formation of ipomeamarone is of particular interest. Because this substance is a unique furanoterpenoid produced by host tissues in response to infection, and because it has remarkable biological properties, such as uncoupling and antipathogenic actions, elucidation of its biosynthetic mechanism would be of great value for the further study of terpene formation and of the host-parasite relationship. Furthermore, an increase of polyphenolic substances, e.g., chlorogenic acid, has been commonly observed in diseased plants (6, 10, 14), and some workers have implied that these play an important role in the defense mechanism, although contradictory evidence has been presented (5, 11). We have undertaken an analytical study of ipomeamarone and chlorogenic acid (3). However, recent chromatographic evidence (1) showing a concurrent formation of many ipomeamarone-like terpenoids in injured root tissues indicates the necessity of a more critical analysis of such metabolites in host tissues. The present paper deals with the quantitative analyses of ipomeamarone and chlorogenic acid in the infected root tissues of resistant and susceptible sweet potato varieties, and their possible role in the defense mechanism.

MATERIALS & METHODS

FUNGUS INOCULATION & PREPARATION OF SAMPLE: Sweet potato roots grown at the farm of the National Agricultural Experiment Station, Konosu, Saitama, were sent to us after harvest in October 1959. They were stored at 10° C until used. During this period neither microbial damage nor sprouting was visible. Varieties Norin no. 1 and no. 10 were selected as exemplifying varieties resistant to the black rot fungus and Norin no. 2 and no. 5 as susceptible ones. The basic procedure for fungus inoculation of root tissues and the subsequent handling was as follows. Individual roots were cut into two pieces lengthwise, and both halves cut lengthwise in 1.5 to 2.0 cm thick slices. One half was the uninoculated control sample; the other half of the slices was treated with a dense water suspension of the black rot fungus prepared from slant cultures, and both groups were then incubated for 5 days at 30° C. The relative humidity was kept 100 % throughout this period. The fungus grew and spread quite uniformly on the root slices and gradual penetration occurred. However, there was a significant difference in the symptoms exhibited by the resistant and susceptible varieties. In the former the successful penetration of fungus was prevented concomitantly with the appearance of the typical necrotic browning of the infected tissue after 72 hours. In the latter case, mycelial growth was more extensive and the necrotic reaction was less pronounced. At 24-hour intervals, infected tissue and uninfected tissue, about 0.5 to 1.0 mm thick adjacent to the infected portion, was harvested for chemical analysis of ipomeamarone and chlorogenic acid. For the analysis of chlorogenic acid in the uninoculated sample, sections 1.0 mm in thickness from the inner part of the sliced tissue were also taken. The thickness of the infected tissue increased as the infection developed: about 0.5 mm at 24 hours, 0.5 to 1.0 mm at 48 hours, and 1.0 mm afterwards in the case of the resistant variety. In the case of the susceptible variety, it was about 0.5 mm at 24 hours and 1.0 mm thereafter. It was desirable to avoid contaminating the adjacent tissues in handling infected and uninfected tissues, but a minor inclusion was technically inevitable. At the 24-hour period the former included a negligible portion of the latter since fungus growth was slight. On the contrary, at the later stages of infection, a slight contamination of the necrotized flecks in uninfected tissue was unavoidable. Thus, a careful interpretation of the analytical data obtained was necessary.

CHEMICAL ANALYSIS: To 0.5 g (fr wt) of each sample was added 5 ml of 95 % ethanol, and the tissue homogenized with the Potter-Elvehjem glass homogenizer. The resulting suspension was gently boiled (with the aid of a reflux condenser) in a hot water bath for 30 minutes, and filtered after cooling. A 0.1 ml aliquot was added to a silica gel chromatostrip with a micro-pipette for both the qualitative examination of component substances and the quantitative estima-

[1] Received July 21, 1960.

[2] This paper constitutes Part 30 of Phytopathological Chemistry of Black-rotted Sweet Potato.

tion of ipomeamarone. Details of the experimental procedure for the chromatostrip technique were given in a previous paper (1, 4). Its application to the quantitative analysis was developed in a manner similar to that of Stanley and Vannier for the analysis of coumarins in citrus oil (9). Since Ehrlich's reaction is rather unstable, a photograph was taken immediately after spraying the color reagent on the strip. In order to determine the magnitude of ipomeamarone synthesis, the portion of the silica gel containing this component was located after the development of strip, eluted with pure methanol, and the carbonyl value determined according to the colorimetric method of Lappin and Clark (7). Its quantity can be calculated from a standard curve obtained from data using pure ipomeamarone. Both pure and crude samples of ipomeamarone used as the reference were prepared according to a previous paper (1).

The ethanol extract was placed on an alumina column and the amount of chlorogenic acid determined by the method of Zucker and Ahrens (15). The calibration curve plotted from data using recrystallized chlorogenic acid and pure isochlorogenic acid was practically the same. The series of analyses of those compounds were carried out using one piece of root per experiment, and quite comparable results were repeatedly obtained. In every experiment analyses were carried out in duplicate and the averaged value presented.

Treatment of Sweet Potato Roots With Poisonous Chemicals: Sliced healthy root tissues were treated with 0.1 % $HgCl_2$ containing 5 % NaCl, or 0.1 % monoiodoacetate following the method of Uritani et al (13). Five per cent NaCl was used as the control reagent. Two additional applications were given 12 and 24 hours later. Forty-eight hours after the first treatment, the tissues, about three to five mm thick, were completely degenerated in the case of the former two chemicals. Both the degenerated and the adjacent tissues were subjected to analysis in the same manner and used above for the infected tissues. NaCl-treated tissues did not show any marked change in appearance.

Results

Chromatographic Study of Ipomeamarone Formation: Figure 1 represents the time-course formation of ipomeamarone and other Ehrlich's reagent-positive substances in infected tissue examined

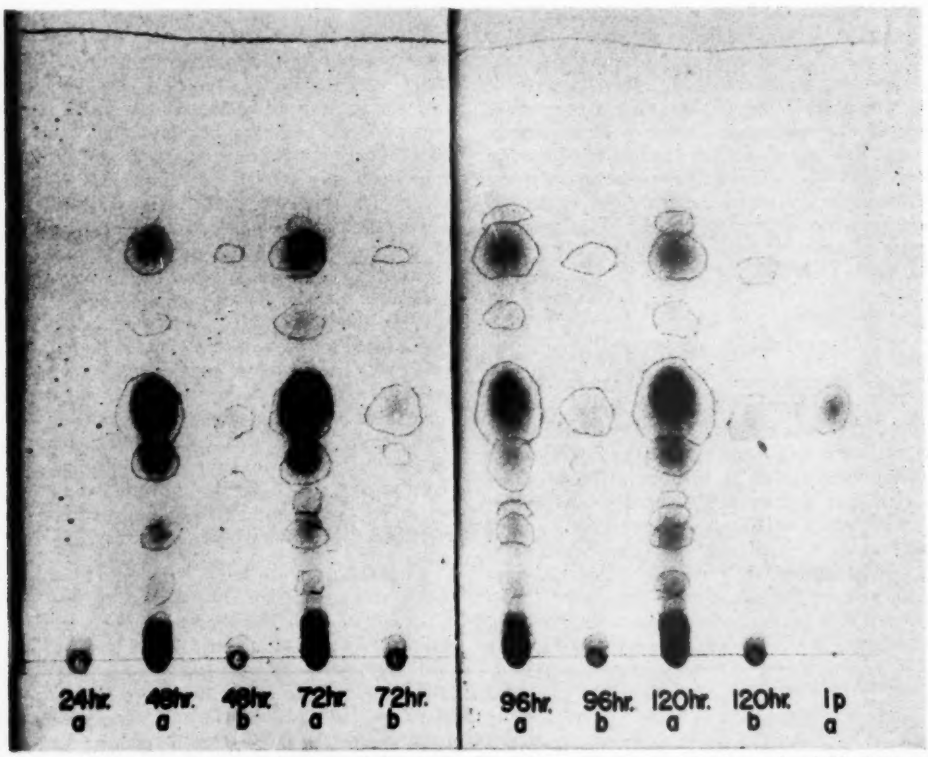

Fig. 1A. Chromatographic examination of the formation of ipomeamarone and other related Ehrlich's reagent-positive substances in sweet potato roots as a function of infection period (hr). (A): Resistant variety Norin no. 1.

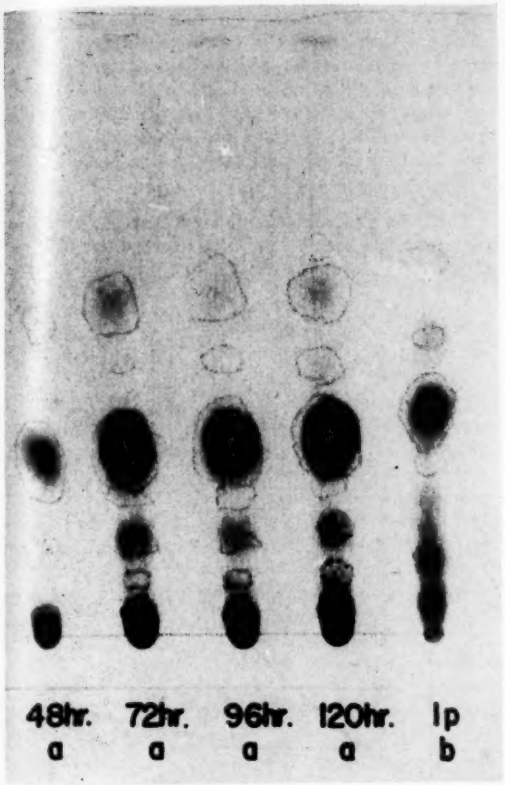

FIG. 1B. Chromatographic examination of the formation of ipomeamarone and other related Ehrlich's reagent-positive substances in sweet potato roots as a function of infection period (hr).

(B): Susceptible variety Norin no. 2. (a) is the infected tissue and (b) the tissue adjacent to this region. Ip. (a) and (b) represent pure and crude ipomeamarone. respectively.

by silica gel chromatostrip. It should be pointed out that, in a qualitative sense, the synthetic pattern of these substances was very similar in both the susceptible and resistant variety. Another important point is that there was almost no visible synthesis of either ipomeamarone or other similar compounds during the initial 24 hours, and a rather striking synthesis in the subsequent 24 hours, thus showing an induction of the drastic metabolic disturbance in host tissues 48 hours after fungus inoculation. After this period little change of the chromatographic picture was seen in either variety. In spite of such similarity in the chromatographic pattern, synthesis of each component in the resistant variety seems to be greater than that in the susceptible one. Formation of Ehrlich's reagent-positive substance in uninfected tissue was very faint and only the results obtained using the resistant variety are shown in figure 1.

QUANTITATIVE DETERMINATION OF IPOMEAMARONE SYNTHESIS: Figure 2 represents a typical result showing the rate of ipomeamarone synthesis in inoculated root tissues. Two points deserve attention. First, in both the resistant and susceptible varieties, there was a lag phase (24 hr) which agrees with the preceding chromatographic evidence. Second, the rate of ipomeamarone synthesis during the two following 24-hour periods was more striking in the resistant than in the susceptible variety. This point is more clearly summarized in table I in which analytical data for four varieties are presented. Synthesis in the highly resistant variety, Norin no. 10,

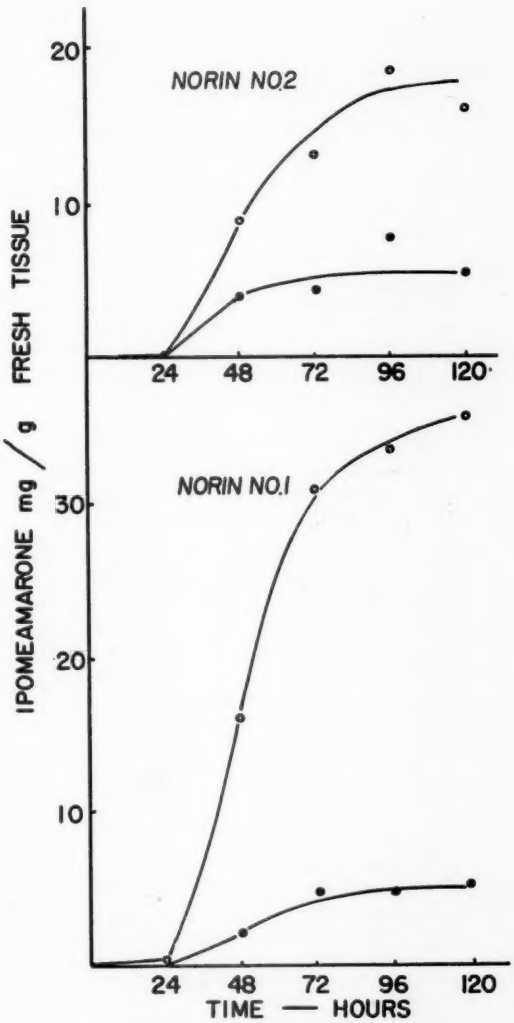

FIG. 2. Synthesis of ipomeamarone in infected tissue (open circle) and adjacent uninfected tissue (closed circle) as a function of infection period (hr).

TABLE I

SYNTHESIS OF IPOMEAMARONE IN INFECTED ROOT TISSUES OF 4 SWEET POTATO VARIETIES AT 2 INTERVALS 24 TO 48 HOURS & 48 TO 72 HOURS

VARIETY	RESISTANCE	IPOMEAMARONE SYNTHESIZED mg/g FR TISSUE	
		24–48 HR	48–72 HR
Norin no. 10	Very high	45.5	14.0
Norin no. 1	High	16.4	14.3
Norin no. 2	Low	8.9	4.3
Norin no. 5	Very low	1.6	1.0

was surprisingly high in the second 24-hour period (from 24 hr – 48 hr), in contrast to an almost negligible synthesis in the least resistant variety, Norin no. 5. The rate of synthesis leveled off somewhat 72 hours after infection, concomitantly with the termination of fungus growth. This event was more or less coincident with the death of the infected root tissues, particularly in the case of the resistant variety, thus the maximum level of ipomeamarone synthesized was significantly higher in the resistant varieties (table I). About three to five milligrams ipomeamarone per gram fresh weight were found in uninfected tissue in both varieties. The ratio uninfected/infected representing the relative amount of ipomeamarone in the adjacent uninfected tissue compared to the infected part, was at the maximum level about one-seventh for the resistant variety and about one-third in the case of the susceptible variety. As discussed above, contamination of infected in uninfected tissue might partially explain such an analytical result, but the formation of ipomeamarone in the healthy tissue in situ would be another possible explanation.

QUANTITATIVE DETERMINATION OF CHLOROGENIC ACID SYNTHESIS: Analytical data for chlorogenic acid synthesis are shown in figure 3. Independent chromatographic experiments have shown that both the chlorogenic acid and isochlorogenic acid content increase in infected roots, in this case the latter compound was found to be the major component (Akazawa, unpubl data). The total amount was expressed as mg chlorogenic acid per g fresh weight in this paper. The concentration of chlorogenic acid in the fresh sweet potato roots was very low, but a significant increase occurred due both to the effect of slicing (wounding) and fungus infection. Three points should be considered. First, throughout the incubation period, the concentration of chlorogenic acid in uninfected tissue was much higher than that in infected, a relationship which was exactly opposite to that observed in the ipomeamarone formation. However, an increase of chlorogenic acid is not a specific reaction of the host tissue induced by the pathogenic infection, because it occurred even in healthy tissues which received a simple mechanical treatment, slicing. Second, unlike ipomeamarone, there was no marked lag phase in the synthesis of chlorogenic acid in uninfected tissue, rather a steady increase was observed. Third, the pattern of chlorogenic acid in the three kinds of tissue samples was quite comparable between two varieties, although the concentration in each tissue was significantly higher in the resistant variety when compared to the susceptible one.

RESPIRATORY INCREASE & IPOMEAMARONE: Ipomeamarone has been found to be a potent uncoupling agent and its role as a respiratory stimulant in in-

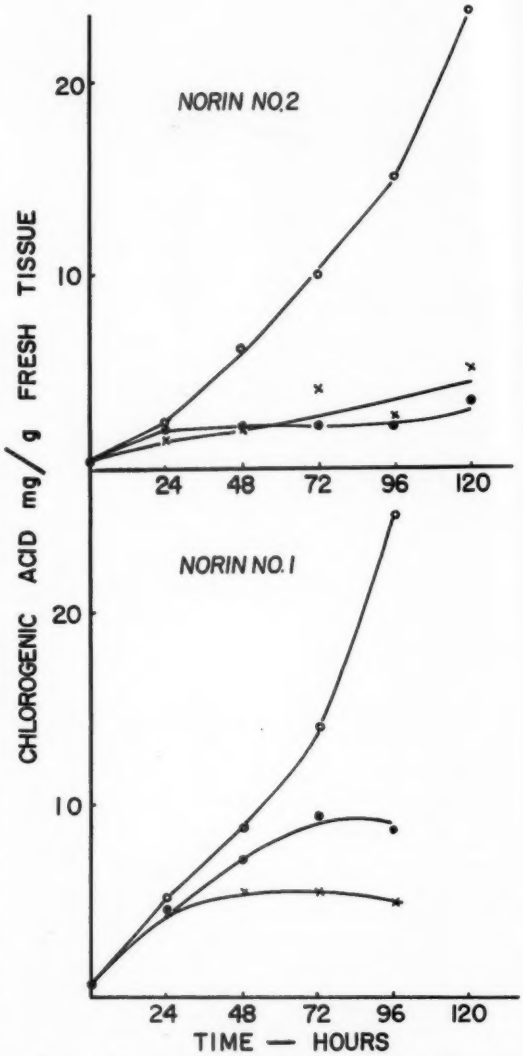

FIG. 3. Synthesis of chlorogenic acid in infected tissue (○——○), tissue adjacent to infected tissue (●——●), and in the sliced healthy tissue (×——×) as a function of infection period (hr).

fected host tissue has been suggested (12). The effect of pure ipomeamarone on the tissue respiration was reinvestigated and an effect similar to that produced by the crude material was found (1). However, the following two points may disprove the hypothesis that ipomeamarone to an appreciable extent is active naturally in infected roots (2, 11); an increase in the respiratory rate is evident in the inner region adjacent to the infected part where no ipomeamarone can be detected, and the respiratory increase begins 24 hours after fungus infection, whereas ipomeamarone synthesis is negligible at this stage even in the infected tissue itself. The analytical results of figure 2 showing the presence of ipomeamarone in the uninfected tissues closely adjacent to the infected tissue do, however, suggest the possible participation of this substance in the respiratory increase in that part. Regarding this point, information obtained in the chemically injured root tissues should be noted. Trends in the respiratory increase similar to that of the fungus-infected tissues were observed in the healthy region of root tissues treated with $HgCl_2$ or monoiodoacetate (13). Chromatographic examination shows a marked synthesis of many substances including ipomeamarone in these tissues (fig 4), and their pattern was practically identical to that of the fungus-infected tissues (cf. fig 1). NaCl treatment, on the other hand, resulted in the formation of a minute amount of Ehrlich's reagent-positive substances which were not clearly detected in the chromatogram. Quantitative estimation of ipomeamarone in the former two cases was not undertaken, but the concentration seemed to exceed that inducing a respiratory increase and was somewhat equivalent to the level exhibiting an inhibitory effect in the in vitro experiment. The question of whether exogenously added ipomeamarone behaves in a similar way to that in living tissues remains open.

Discussion

Uritani and Akazawa (11) have suggested that the pathological disturbance of carbohydrate and fatty acid metabolism in the host may induce the synthesis of ipomeamarone, an abnormal sesquiterpenoid. It has been postulated that its biosynthesis involves the incorporation of mevalonic acid as a precursor (13). Concurrent formation of many substances in the infected tissues is an important finding in this respect and its possible connection to the biosynthesis of ipomeamarone has been discussed (1). It cannot be overemphasized that the present analysis has uncovered a drastic synthesis of ipomeamarone in host tissues only after a lag of 24 hours after fungus infection. The infected region may not be a suitable material for the bio-synthetic study, however, because it is composed of two different systems, host tissues and the pathogenic microorganism. However, chemically-treated sweet potato tissues which form a significant amount of ipomeamarone in their healthy region may offer an alternative way to elucidate this problem. The information gained in this work supports the view that this substance is synthesized by the root tissue per se.

It was found previously that ipomeamarone has several antibiotic properties against the black rot fungus; inhibition of growth, protein synthesis, phosphate metabolism, and respiration among others. Thus its fungitoxic action in host tissues is quite clear (11). Along with this evidence, it should be emphasized that there exists a close correlation between the amount of ipomeamarone synthesized in the host and the degree of host resistance against the pathogen. Müller has proposed a function for phytoalexin in the defense mechanism of host plants based on finding the formation of some organic substance having antipathogenic activity in the inner epiderms of pea pods infected with *Sclerotinia fructicola* and *Phytophthora infestans* (8). In a pathological sense, mode of action of ipomeamarone seems to be quite similar to that of the Müller's phytoalexin, and its physiology warrants further experimentation.

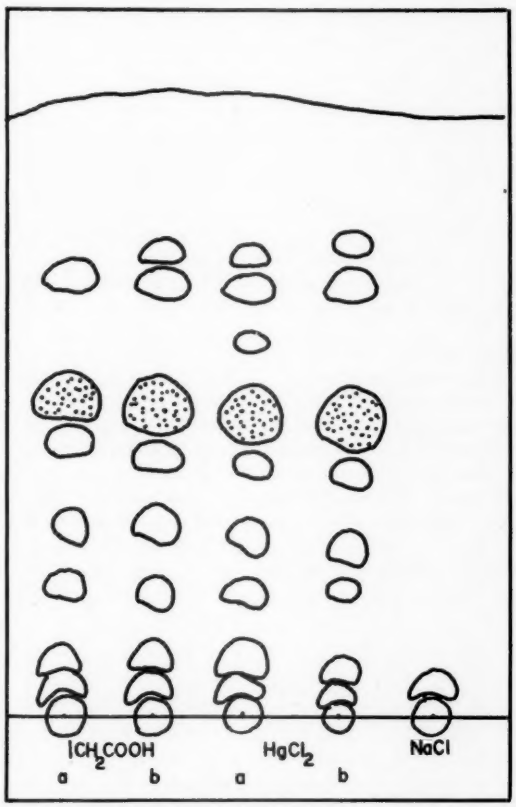

Fig. 4. Chromatographic examination of the formation of ipomeamarone and other related substances in the chemically treated root tissues after a 48-hr period, (a) and (b) represent degenerated and adjacent tissue, respectively. Ipomeamarone was marked by spots.

Turning to the antipathogenic function of polyphenolic substance, conclusive evidence for their participation is lacking so far, although many workers have reported a remarkable synthesis of these compounds in diseased plants (6, 10, 14). Present data does not provide any favorable information whatsoever, but it would be of interest to investigate the nature of chlorogenic acid synthesis as a different approach to the study of metabolic alteration.

Though several hypotheses have been put forward, the crucial mechanism in the respiratory increase in infected plant tissues remains unclear (11). Analytical data shown in figure 2 may indicate a minor participation of ipomeamarone in the respiratory increase of tissue closely adjacent to the infected region, but this is obviously not the case during the very early stages of infection. In fact, independent isotopic work using C^{14}-labeled glucose has given evidence that two different mechanisms are operating in the respiratory system of sweet potato roots with black rot (Akazawa, unpubl data). The respiratory increase in the chemically injured tissues may presumably be attributed to the uncoupling effect of ipomeamarone and the mechanism appears to be different from that of the infected tissues.

Summary

The formation of ipomeamarone and other related substances was compared by silica gel chromatostrip technique in two sweet potato varieties, one resistant the other susceptible to black rot. Chromatographs showing the synthetic pattern were practically the same for both varieties. When ipomeamarone synthesis was quantitatively analyzed, it was found that synthesis occurred quite strikingly after a lag phase of 24 hours and reached a maximum level at about 72 hours after infection. The rate of synthesis and the maximum level in the infected tissues were higher in the resistant than in the susceptible variety, thus the possible function of the substance in the defense mechanism of host tissues was inferred. A steady increase in chlorogenic acid content was found in uninfected tissue adjacent to infected tissues, which was an entirely different picture from that of ipomeamarone. Findings that its increase was induced even by slicing and that no significant difference in the distribution existed between two varieties may refute the primary function of this compound in the defense mechanism of the host. The role of ipomeamarone in the respiratory increase of infected root tissue was discussed from the analytical data of both infected and chemically treated root tissues.

Acknowledgment

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SOME EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID ON SOLUBLE NUCLEOTIDES & NUCLEIC ACID OF SOYBEAN SEEDLINGS [1, 2]

JOE L. KEY [3] & JOHN B. HANSON

One of the most marked biochemical changes in plants known to accompany 2,4-D[4] treatments is the increase in nucleic acid. Rebstock et al (33) found the nucleic acid content to double in the stems of bean plants after 2,4-D treatment. Similar increases in the RNA content of 2,4-D-treated cucumber plants were reported by West et al (40); 2,4-D treatment was also shown to alter the RNA content of excised tissue both in cucumber and in corn (40). It has been proposed that auxin action is linked to nucleic acid metabolism (36), and that an alteration of nucleic acid metabolism is involved in the abnormal growth and development of 2,4-D-treated plants (33). Work by Marrè and Forti (24) has shown that auxins initiate a large increase in ATP in pea stem sections within 30 minutes after treatment. More recently Ormrod and Williams (29) have shown that 2,4-D treatment causes a rapid rise in soluble organic phosphates, suggesting an increase in such compounds as ATP (since inorganic phosphate is usually incorporated into organic compounds via oxidative phosphorylation). RNA is apparently involved in such processes as protein synthesis (3), oxidative phosphorylation (12), and ion absorption (19, 38), processes known to be affected by 2,4-D (9, 16, 28, 35, & 37). Soluble nucleotides are involved in such essential biological processes as oxidative phosphorylation (20), amino acid metabolism (8), carbohydrate transformations (14), and lipide syntheses (15). Therefore it seemed that additional basic information on the effects of 2,4-D on nucleotide metabolism was needed.

These data are results of experiments undertaken to study some relationships of 2,4-D to nucleotide metabolism. The results suggest that changes in nucleotide metabolism may underlie the growth aberrations induced by 2,4-D. However, more basic knowledge of the normal growth processes will be necessary before the role of 2,4-D as an auxin or as a herbicide can be fully clarified.

Materials & Methods

Soybean seeds (Hawkeye variety) were lightly dusted with the fungicide Spergon (U.S. Rubber Co.) and planted in 8.5 × 13 inch pyrex baking dishes between layers of vermiculite moistened with tap water and germinated at 28° C in the dark. In experiments where whole plants were used, the seedlings were sprayed with 15 ml of 5×10^{-4} M 2,4-D per tray of seedlings (ca. 200 plants) after 60 hours of germination. The 2,4-D was twice recrystallized from ethanol and placed in solution as the potassium salt (pH 6.0). Comparable seedlings which were not sprayed with 2,4-D were used as controls in all experiments. In studies where sections of hypocotyl were used, the sections were cut from seedlings which had been germinated for 72 hours as described above.

Unless otherwise stated in figures and tables, sections of hypocotyl tissue were incubated in a solution consisting of 0.002 M potassium dihydrogen phosphate (neutralized to pH 6.0 with ammonium hydroxide), 0.5 percent sucrose, and 0, 10, or 500 ppm 2,4-D.

For analysis of protein and nucleic acid and estimates of soluble nucleotide, tissue samples were ground in an ice-jacketed Potter-Elvehjem tissue homogenizer for 2 to 3 minutes in 5 ml of 0.5 M sucrose. The cell debris and nuclei were removed by centrifuging the homogenate at 1000 × g for 5 minutes at 0° C. Aliquots of the cleared homogenates were treated with equal volumes of ice-cold 10 % trichloroacetic acid, and the precipitate was sedimented by centrifuging at 2000 × g for 5 minutes. The pellet was washed by resuspension and resedimentation in 10 % trichloroacetic acid, dissolved in 0.1 N NaOH, and aliquots were analyzed for protein by the method of Lowry et al (21).

Additional aliquots were removed from the cleared homogenate and treated with an equal volume of 0.4 N perchloric acid for nucleic acid and nucleotide analyses. The precipitate was sedimented as above, and the pellet was washed by resuspension and resedimentation in 0.2 N perchloric acid. The combined supernatant solutions were used for estimation of soluble nucleotide by referring the 260 to 290 mμ absorption difference to an AMP standard curve. The centri-

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[2] Contribution from the Department of Agronomy, University of Illinois. This research was supported in part by Grant G-3358, National Science Foundation and represents a portion of a thesis submitted by J. L. Key for the Ph.D. degree.

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[4] Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; RNA, ribonucleic acid; ADP, adenosinediphosphate. Other abbreviations as footnotes in tables.

fuge pellet was next extracted twice with 2-ml portions of ethanol-ether-chloroform (2:2:1) to remove lipides. The defatted pellet was extracted with 0.5 N perchloric acid for 30 minutes at 70° C, centrifuged, and the supernatant solution used for determining RNA by referring the 260 to 290 mμ absorption difference to a standard curve with yeast RNA.

In experiments where ADP-C^{14} incorporation into RNA was studied, 3 g of the designated tissue were incubated at 28° C in 20 ml of solution (see footnote to table III) containing 1 μc of ADP-8-C^{14} (1.25 μc/mg ADP). After the tissue sections were blotted and weighed, the samples were homogenized in deionized water, made to a volume of 20 ml, filtered through glass wool and centrifuged for 5 minutes at 1000 × *g*. A 0.1 ml aliquot of the supernatant fluid was removed and plated for determination of total ADP uptake. (No attempt was made to determine what form the C^{14} entered the tissue, but is assumed to be ADP-C^{14}.) Additional aliquots were removed, and the procedures described above were followed for RNA extraction. One milliliter of the KOH-neutralized perchlorate-free (potassium perchlorate being relatively insoluble at 0° C) extract was plated for determining ADP incorporation into RNA. Counting efficiency was routinely checked by adding known amounts of radioactivity as ADP-C^{14} to comparable aliquots from "cold" tissue homogenates. The efficiency of counting was 10 % whether made on the crude supernatant for total uptake or on the RNA extract for incorporation.

A more exact analysis for soluble nucleotides was made by the procedures of Cherry and Hageman (6). Briefly the methods were as follows: Sixty-gram samples of hypocotyl tissue were homogenized for 4 minutes in an Omnimixer in 120 ml of cold 0.6 N perchloric acid. The homogenate was centrifuged at 20,000 × *g* for 15 minutes at 0° C. The supernatant solution was filtered through glass wool and neutralized to pH 6.8 with cold 4.0 N KOH. After 30 minutes at 4° C the solution was filtered, and the perchlorate-free extract was placed on a column (1.2 × 40 cm) of Dowex-1×10 (200–400 mesh, formate form) ion-exchange resin. Nucleotides were eluted from the column by means of a formic acid gradient followed by a formic acid-ammonium formate gradient. The eluate was collected in 5 ml volumes in a Rinco automatic fraction collector. The concentration of nucleotide was estimated by measuring the 260 mμ absorption of the contents in each tube in a Beckman DU spectrophotometer.

The contents of all tubes from each peak were combined and purified for identification of the component nucleotides. Co-chromatography on paper in Pabst solvents (31) and ultraviolet absorption characteristics were used to identify the components present in each purified fraction. Standard nucleoside-5′-phosphates were co-chromatographed with each unknown fraction. The nucleotides were located on the developed chromatograms with a Mineralight lamp. The ultraviolet-quenching spots were eluted with sodium phosphate buffer from the chromatograms which had been developed in Pabst solvent III, and the ultraviolet absorption spectra were read between 220 and 300 mμ at pH 2, 7, and 11 for both known and unknown compounds. In addition 2 mg each of standard Pabst AMP, GMP, UMP, ADP, UDP, and ATP (5′-phosphates) were added separately to extracts of the hypocotyl tissue and column chromatographed to confirm the elution position of these compounds. The presence of DPN was confirmed by adding sodium hydrosulfite to the fraction which was believed to contain DPN, the oxidized DPN being reduced to DPNH which gave a second absorption peak at 340 mμ.

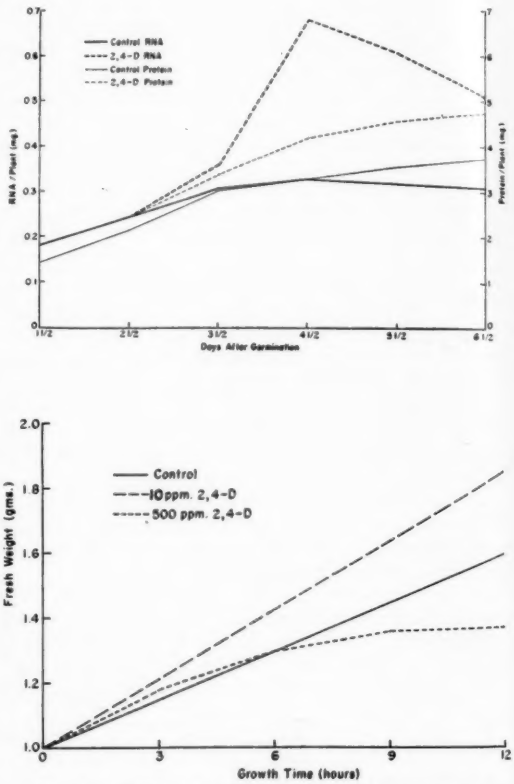

FIG. 1. Changes in RNA and protein accompanying normal and 2,4-D-induced growth of soybean seedlings. Analyses were made on triplicate samples.

FIG. 2. Growth of excised soybean hypocotyl sections accompanying 2,4-D treatment. 1-cm hypocotyl sections were taken just back of the cotyledons and incubated in a solution consisting of 0.002 M NH_4KPO_4 (pH 6.0), 0.5 % sucrose and 0, 10, or 500 ppm 2,4-D. Fresh weights were measured at 3 hour intervals for 12 hours. Values represent averages from two experiments each with duplicate samples.

TABLE I

DISTRIBUTION OF NUCLEIC ACID & PROTEIN IN SOYBEAN HYPOCOTYL AS FUNCTION OF MATURITY & 2,4-D TREATMENT*

SECTION	TREATMENT	RNA		PROTEIN		SOLUBLE NUCLEOTIDE	
		Mg/g F.W.	Mg/ SECTION	Mg/g F.W.	Mg/ SECTION	Mg/g F.W.	Mg/ SECTION
0.0–0.5 cm	Control	2.80	0.038	30.7	0.43	0.44	0.006
	2,4-D	3.07	0.052	31.6	0.53	0.49	0.009
0.5–1.5 cm	Control	1.29	0.042	15.4	0.51	0.31	0.013
	2,4-D	0.76	0.058	8.4	0.64	0.26	0.020
1.5–2.5 cm	Control	0.58	0.020	6.6	0.23	0.21	0.007
	2,4-D	0.56	0.038	5.5	0.37	0.22	0.015
2.5–3.5 cm	Control	0.42	0.016	5.6	0.22	0.18	0.007
	2,4-D	0.80	0.096	6.8	0.38	0.24	0.014

* Successive sections of 3.5-day-old control and 2,4-D-treated soybean hypocotyls were taken for analyses beginning just back of the cotyledons. 2,4-D-sprayed plants were treated at 2.5 days. Data are averages of two closely duplicating experiments.

RESULTS

PROTEIN & NUCLEIC ACID: The general morphological and growth responses to 2,4-D (5×10^{-4} M in the experiments reported) were essentially those often reported, and are described in a previous paper (16). Elongation of root and shoot of treated intact plants was almost completely inhibited, apparently due to inhibition of both cell division and cell elongation (34). Normal meristematic activity was resumed, at least in part, above and below the cotyledons between 2 and 3 days after treatment with 2, 4-D. Root growth which is dependent upon food reserves from the cotyledons (39) did not show this recovery. Disorganization of the phloem resulting from uncontrolled cell division (10, 34) might account for lack of recovery in the root tissue. Tissue proliferation in the lower hypocotyl and root became apparent between 48 and 72 hours after treatment with 2,4-D.

The changes in protein and nucleic acid per plant (less cotyledons) accompanying 2,4-D-induced growth abberations (fig 1), are in general agreement with published results (33, 35, 40). The changes with time are of special interest here. After 3.5 days of growth there were no marked changes in protein or RNA in control seedlings, although the slight increase in protein and decrease in RNA were found reproducible. In contrast the RNA content doubled in 2,4-D-treated plants between 24 and 48 hours after treatment. The peak in RNA content occurred just prior to the time proliferation in the lower hypocotyl became apparent, suggesting a rather direct interaction of 2,4-D-induced RNA synthesis and cell proliferation. The increase in RNA seems not to be a simple increase in cytoplasm since the protein/RNA ratio decreased very markedly in treated seedlings, especially at 48 hours after treatment. As the plants recovered (4.5–6.5 days), there was a noticeable shift in the protein/RNA ratio back toward normality. Similar analyses were made separately on hypocotyl and root tissue and revealed that the effects were obtained in both tissues, although the changes were of greater magnitude in the hypocotyls (18).

It was of interest to attempt to localize the RNA changes so far as stage of cell development was concerned. In order to do this sections were cut successively from the hypocotyls, starting with the hypocotyledonary hook as the 0.0 to 0.5 cm section and proceeding down the hypocotyl to obtain sections with increasing mean cell age. As shown in table I the largest increase in RNA occurred in the mature tissue (2.5–3.5 cm) which would have rapidly proliferated in another 24 to 48 hours. The increase in RNA in the first three sections (0–2.5 cm) was essentially proportional to the radial enlargement which these cells had undergone. The increase in soluble nucleotide which occurred following 2,4-D-

TABLE II

FRESH WEIGHT, NUCLEOTIDE, NUCLEIC ACID, & PROTEIN CHANGES ACCOMPANYING GROWTH OF EXCISED SOYBEAN HYPOCOTYL SECTIONS*

TREATMENT	INCREASE IN FRESH WT %	RNA Mg/g F.W.	PROTEIN Mg/g F.W.	SOLUBLE NUCLEOTIDE Mg/g F.W.
Initial	...	2.35	19.1	0.47
Control	53.7	2.07	17.4	0.41
10 ppm 2,4-D	86.2	2.05	17.9	0.40
500 ppm 2,4-D	38.0	2.19	18.9	0.34

* Details of experiments are given in footnote of figure 2. Average of three experiments.

treatment is of interest and will be discussed more fully in the material which follows.

Similar studies were extended to excised tissue-section growth where West et al (40) had also shown 2,4-D to affect RNA metabolism. Figure 2 shows the effect of 10 and 500 ppm 2,4-D on the growth of the excised hypocotyl tissue. (The apical cm section of the hypocotyl was used in these experiments.) The initial promotion of growth by 500 ppm 2,4-D (small but very reproducible) followed by complete inhibition is of interest. Growth stimulation followed by inhibition as a result of auxin treatment has been reported previously (1, 23, 30), and West (41) reported a comparable stimulation and inhibition of respiration. As shown in table II, growth of excised hypocotyl sections was associated with the catabolism of both RNA and protein. The growth-promoting concentration of 2,4-D had no observed effect on RNA and protein metabolism, whereas the inhibitory concentration of 2,4-D either blocked this catabolism or induced a greater resynthesis of these constituents. The presence of ammonium ion and sucrose would provide materials for synthesis. Solutions remained clear with no evident bacterial contamination.

Some preliminary experiments were run in an attempt to see if herbicidal concentrations of 2,4-D had inhibited the normal catabolism of RNA or if the synthetic processes had been speeded up relative to the breakdown. Excised sections were incubated in ADP-C^{14} in the presence of 0, 10, and 500 ppm 2,4-D, and the incorporation of ADP into acid-insoluble nucleic acid was measured, (table III).

Experiment A was run under conditions about comparable to those of table II; that is with 0.002 M

TABLE III

EFFECT OF 2,4-D ON INCORPORATION OF ADP-C^{14} INTO RNA OF EXCISED SOYBEAN HYPOCOTYL TISSUE*

TREATMENT & TISSUE	% INCREASE FR WT	SOLUBLE NUCLEOTIDE	RNA	ADP-C^{14} UPTAKE & INCORPORATION		
		mg/g Fr Wt		c/m/g Fr Wt UPTAKE	c/m/g Fr Wt RNA	% INCORPORATION
EXPERIMENT A						
Tip—initial	...	0.44	2.75	...	...	...
Control	45.7	0.43	2.28	14,580	1,790	12.3
10 ppm 2,4-D	71.0	0.40	2.57	16,060	2,080	12.5
500 ppm 2,4-D	35.7	0.37	2.66	9,760	815	8.4
Base—initial	...	0.23	0.57	...	...	...
Control	3.4	0.20	0.58	6,020	790	13.2
10 ppm 2,4-D	10.3	0.23	0.66	6,830	1,710	25.0
500 ppm 2,4-D	0.7	0.19	0.60	5,010	415	8.3
EXPERIMENT B						
Tip—initial	...	0.45	3.02	...	...	...
Control	54.2	0.39	2.25	17,700	2,440	13.8
10 ppm 2,4-D	70.0	0.42	2.20	18,800	3,310	17.6
500 ppm 2,4-D	22.9	0.24	2.13	12,950	2,490	19.2
Base—initial	...	0.20	0.56	...	...	...
Control	7.9	0.20	0.56	12,725	2,380	18.8
10 ppm 2,4-D	16.0	0.20	0.60	14,400	3,775	26.0
500 ppm 2,4-D	3.0	0.11	0.62	12,200	4,900	39.5
EXPERIMENT C						
Control tip —I	...	0.48	3.11	...	...	...
F	35.2	0.38	2.59	17,450	2,182	11.9
2,4-D tip —I	...	0.54	3.52	...	...	...
F	18.5	0.45	3.04	14,170	1,645	11.8
Control base —I	...	0.22	0.57	...	...	...
F	6.2	0.20	0.56	6,377	688	11.0
2,4-D —I	...	0.23	0.42	...	...	...
F	22.3	0.21	0.55	7,035	1,248	16.9

* Conditions for experiment A: 3 g of tissue were incubated for 10 hours in a solution containing 1 % sucrose, 0.002 M KH_2PO_4 buffer neutralized to pH 6 with NH_4OH, 1 μc ADP-8-C^{14}, and 0, 10, or 500 ppm 2,4-D as designated. Conditions for experiment B were same as for A except that tissue was incubated for 15 hours in 0.004 M buffer. Tip and base refer to the tip 1 cm of the hypocotyl just below the cotyledons and the 2 to 3 cm-section, respectively. In experiment C, the tissue was incubated under identical conditions to experiment B except that the solution contained no 2,4-D. 2,4-D in the table refers to sections which were excised from plants which had been sprayed 24 hours previously with 5×10^{-4} M 2,4-D as described in text. I and F refer to samples analyzed at beginning of experiment and after incubation, respectively. Data are averages of two closely duplicating experiments.

potassium dihydrogen phosphate buffer (neutralized to pH 6 with ammonium hydroxide) and for 10 hours. Under these conditions, 2,4-D maintained or increased the level of RNA in the tissue. The growth-promoting concentration of 2,4-D enhanced the absorption of ADP relative to the control, while the herbicidal concentration depressed absorption. The proportion of the absorbed ADP incorporated into RNA was increased by 10 ppm 2,4-D in the basal section, but not in the tip section. The high concentration of 2,4-D not only inhibited the absorption of ADP, but also inhibited the incorporation into RNA of that which was absorbed.

As shown in experiment B, however, if the buffer concentration is doubled and the time of incubation extended to 15 hours, a markedly different result is obtained. Here the herbicidal concentration of 2,4-D increased the percentage of absorbed ADP which was incorporated into RNA. Since it appears that high salt concentrations can accelerate RNA metabolism by endogenous enzymes (13), the different result may come largely from the increased buffer. A detailed study will be necessary to clarify these divergent results.

To obtain information relative to ADP incorporation under conditions more nearly comparable with those in the rest of this investigation, tip and basal hypocotyl sections were excised from control and 2,4-D-sprayed plants, and analyzed for nucleotides before and after floating for 10 hours on 0.002 M buffer containing C^{14}-labeled ADP. In the tip section the 2,4-D pretreatment decreased the amounts of growth and ADP absorbed. The percentage of absorbed ADP entering the RNA was unchanged, however. In the basal section, the 2,4-D pretreatment increased expansive growth, ADP absorbed, and the percentage incorporated into RNA. In addition, this section had a net increase in RNA, just as would have occurred in situ (fig 1). The sucrose and ammonium ion probably did not supply needed substrate as well as translocation from the cotyledons, thus reducing the absolute gain in RNA.

As has already been pointed out (40), these data show definite differences between the effects on nucleic acid metabolism induced by 2,4-D in intact plants and in excised plant parts. The data also indicate that 2,4-D has some marked and basic effects on nucleic acid metabolism and on the acid-soluble nucleotide fraction of 2,4-D-treated sections and seedlings. Since alterations of nucleotide metabolism seemed basic to the response to 2,4-D, it seemed of considerable interest to characterize the soluble nucleotide changes which accompanied 2,4-D treatment.

SOLUBLE NUCLEOTIDE: Since the separation and identification of soybean seedling nucleotides had apparently not been done, it was necessary to first separate and identify the component nucleotides of normal soybean seedlings before being able to evaluate the changes induced by 2,4-D.

The elution chromatograms obtained from mature

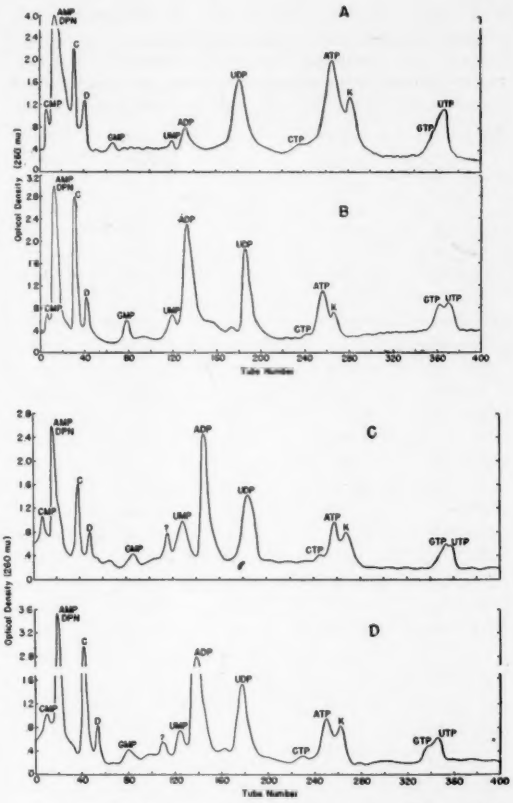

FIG. 3. Elution chromatogram of nucleotides. A. From 50 g of mature, hydrated soybean seed. B. From 50 g of control 2.5-day-old soybean hypocotyl.

FIG. 4. Elution chromatogram of nucleotides from 50 g of 3.5-day-old soybean hypocotyls. C. Control. D. 2,4-D-treated.

soybean seeds and soybean hypocotyl tissue (control & 2,4-D-treated) are shown in figures 3, 4, and 5. Excellent separation of the nucleotide components with symmetrical peaks was obtained in most cases. The data presented in table IV were used as the primary means of identification of the individual nucleotides so designated in figures 3, 4, and 5. In addition to the data shown in table IV similar ultraviolet absorption data were obtained at pH 2 and pH 11 and mobility characteristics in other solvents checked. These data are not reported because of complete agreement with the data shown in table IV. In the case of ATP identification, enrichment of an extract with 2 mg of adenosine-5′-triphosphate was the only technique used which clearly distinguished between adenosine-5′-triphosphate and the component present in peak K. Otherwise identical data suggest that the component present in this fraction may be another form of ATP. In addition to the identification of

TABLE IV

IDENTIFICATION OF NUCLEOTIDE COMPONENTS FROM ELUTION CHROMATOGRAMS*

COMPOUND		R.F.**	250/260***	280/260	λ MAX.
CMP	K	0.70	0.85	0.95	273
	UK	0.69	0.87	0.91	275
AMP	K	0.22	0.78	0.12	260
	UK	0.24	0.77	0.17	260
DPN	K	0.24	0.76	0.09	260
	UK	0.27	0.81	0.08	260
GMP	K	0.38	1.13	0.67	252
	UK	0.38	1.10	0.63	252
UMP	K	0.66	0.67	0.34	262
	UK	0.67	0.69	0.37	262
ADP	K	0.32	0.74	0.11	260
	UK	0.31	0.76	0.12	260
UDP	K	0.74	0.70	0.34	262
	UK	0.73	0.71	0.32	262
CTP	K	0.80	0.86	1.37	275
	UK	0.78	0.88	1.40	274
ATP	K	0.37	0.78	0.14	260
	UK	0.37	0.80	0.17	260
"K"	UK	0.36	0.79	0.20	260
GTP	K	0.52	1.14	0.66	252
	UK	0.53	1.12	0.64	252
UTP	K	0.73	0.76	0.40	261
	UK	0.72	0.77	0.43	262

* C, A, G, and U represent cytidine, adenosine, guanosine and uridine. respectively. MP, DP, and TP represent mono-, di-, and triphosphate nucleotides, respectively. DPN is diphosphopyridine nucleotide.

** R.F.'s were determined in Pabst Solvent III using authentic samples of nucleoside-5′-phosphates (K) and material from the designated peaks (UK).

*** All optical density values reported were determined at pH 7.0 from eluates of nucleotide spots from chromatograms developed in solvent III.

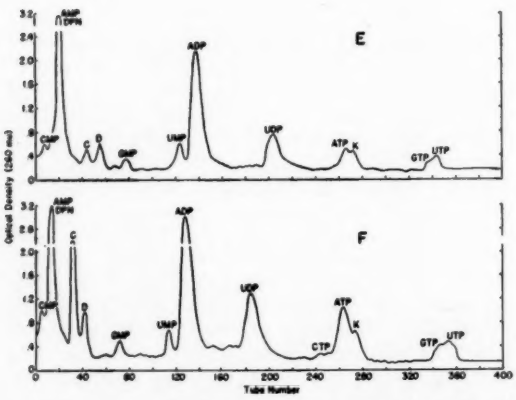

FIG. 5. Elution chromatogram of nucleotides from 50 g of 4.5-day-old soybean hypocotyls. E. Control. F. 2,4-D-treated.

the nucleotides shown in table IV, the component present in peak C of the elution chromatograms has been recently identified as ascorbic acid (17). The component in peak D is an artifact of isolation in that it represents a breakdown product of ascorbic acid (18).

The data indicate a general increase in soluble nucleotides in 2,4-D-treated seedlings and a maintenance of this high level, whereas the quantity of nucleotide decreased with age of control seedlings. The changes noted in adenine nucleotides and ascorbic acid deserve special attention. Quantitative changes in ADP and ATP are shown in table V. Within 3 hours after treatment with 2,4-D the ATP content of the hypocotyl had increased about 45 %. Apparently this increase in ATP was not associated with a corresponding decrease in AMP and ADP. Although the quantity of AMP is not shown in table V because of the presence of DPN in the same fraction, recent work (17) has shown that the oxidized pyridine nucleotide content does not change with 2,4-D treatment, thus it can be concluded that 2,4-D had no effect on the concentration of AMP (figs 3, 4, & 5). The increase in ATP noted here is consistent with

TABLE V

EFFECT OF 2,4-D SPRAY TREATMENT ON LEVEL OF ADENINE NUCLEOTIDES IN SOYBEAN HYPOCOTYLS*

TREATMENT	O.D. UNITS/50 g TISSUE**		
	ADP	ATP	TOTAL
Control (63 hr old)	106	47	153
2,4-D (63 hr old)	106	68	174
Control (84 hr old)	90	33	123
2,4-D (84 hr old)	130	47	177
Control (108 hr old)	93	10	103
2,4-D (108 hr old)	147	55	202

* Absorbance read in $HCOONH_4$-HCOOH. The base line absorption was subtracted from each reading before the units of nucleotide were calculated. One O.D. unit is that amount of nucleotide which will give unit optical density in 1 ml of solution in 1 cm Beckman cuvettes. Values reported are averages from closely duplicating experiments.

** O.D. values determined at 260 mμ.

the work of Marrè and Forti (24), which showed a large increase in ATP in auxin-treated pea stem sections shortly after treatment. The high level of ATP was maintained for only a short time, again in agreement with the work of Marrè and Forti.

The increase in ascorbic acid (peak C) following 2,4-D treatment (figs 4 & 5) has been discussed in greater detail elsewhere, (17). However, in view of the work of Marrè et al (24, 25) and Prochazka's group (from Bentley, 2), the results obtained here are suggestive of a close relationship between auxin action and ascorbic acid metabolism.

Discussion

The data reported here lend further support to the view that the ultimate action of auxin may be related to nucleotide-nucleic acid metabolism. The major quantitative changes in RNA (fig 1) occur several hours after some effects of 2,4-D have become apparent. However, with the techniques used, only quantitative changes in RNA were measured. It is possible that 2,4-D may initiate changes in specific kinds of metabolically active RNA which in turn could mediate auxin effects. More refined techniques are obviously needed to adequately characterize the RNA from auxin-treated tissue. The results do strongly suggest that the 2,4-D-induced increase in RNA underlies the abnormal proliferation of the mature tissue. The experiments using ADP-C^{14} also show that 2,4-D induces changes in RNA metabolism which are not necessarily reflected in quantitative changes (see table III). Recently, Masuda (27) has proposed that auxin promotes cell elongation by causing an increase in the cation binding capacity of RNA at the protoplasmic surface which would allow for loosening of cell walls because of calcium removal from cell wall pectic substances. As already pointed out, Skoog (36) has proposed a mechanism of auxin action involving nucleic acids. Certainly, more work is needed to clarify possible auxin-nucleic acid relationships as a mechanism of auxin action.

In addition to the effects of 2,4-D on RNA, certain changes in the soluble nucleotide fraction were noted. In general, the 2,4-D treatment caused the maintenance of a higher concentration of many of the nucleotides (see figs 4 & 5). Of most interest are the changes in adenine nucleotides noted in table V. In view of the early increase in ATP noted in table V and the data of Marrè and Forti (24), an explanation of the respiratory burst following auxin treatment based solely on activation of endergonic processes which utilize ATP thus giving more adenylate acceptor (4, 5,11) seems to need some modification. As pointed out by Marrè and Forti (24), the shift in the ATP-ADP ratio indicates first an increase in ATP as a result of activation of oxidative phosphorylation followed, in turn, by activation of endergonic processes. Marrè et al (24, 25) attribute this activation either to the effect of auxin on the oxidation-reduction state of the ascorbic acid-glutathione system or to a direct interaction of auxin with the dehydrogenase enzymes. In view of the effects of 2,4-D on increasing the concentration of ascorbic acid and soluble and protein sulfhydryl in soybean seedlings (17), such an activation of the respiratory system is conceivable.

Another possible explanation of the auxin-induced respiratory burst could lie at the adenylate level. If it is assumed that the level of adenylate acceptor normally limits respiration (4, 5, 11), an increase in adenine nucleotide would presumably result in an enhanced rate of respiration. The data reported in table V and figures 4 and 5 suggest that such an increase has occurred. It has been reported previously (16) that mitochondria isolated from 2,4-D-sprayed soybean seedlings contain considerably more soluble nucleotide than mitochondria from comparable control tissue; also, the oxygen uptake by isolated mitochondria from 2,4-D-treated seedlings was not enhanced by adding AMP.

Summary

Some studies on the effects of 2,4-D on soluble nucleotide and nucleic acid metabolism of soybean seedlings and excised hypocotyl sections are reported. Large increases in RNA content of treated plants were found, the maximum concentration being obtained just prior to initiation of cell proliferation in the more mature tissue. The protein-RNA ratio decreased markedly up to 48 hours after treatment, after which time there was a decrease in RNA and an increase in the ratio. Alterations of ADP-C^{14} incorporation into RNA were effected by 2,4-D treatment.

Treatment with 2,4-D also induced an increase in acid-soluble nucleotides of soybean seedlings. These nucleotides were separated and isolated by ion-exchange chromatography and identified. Marked changes in adenine nucleotides were obtained, and these changes are discussed in relation to auxin-induced respiratory increases.

Some changes in ascorbic acid concentration following 2,4-D treatment are discussed.

Acknowledgment

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BASIS OF SHOOT RESPONSE TO ROOT TEMPERATURE IN TOMATO[1, 2]

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Shoot growth of young tomato plants increases as root temperature increases over the range 10° to 25° C (29, 38). When roots are subjected to a temperature of 10° C, the growth rate of shoots approaches zero, even though the shoots are exposed to normal greenhouse temperatures. Rapid shoot growth due to increased root temperature is accompanied by increased concentrations of potassium and phosphorus in the shoot. Concentrations of calcium and magnesium reach maxima at approximately 20° C. Similar variations in mineral concentrations are found in a few other species (35), but are by no means exemplary for plants in general (2, 23, 33, 39).

In the case of the tomato, it might be proposed that reduced rates of mineral transport are responsible for slow shoot growth at cool root temperatures. On the other hand, slow growth might be due to the depressing effect of cool root temperature on the water absorption of plants. The rate of nitrate reduction has also been considered as a possible factor.

This paper reports experiments designed to explore the basic nature of this phenomenon. The evidence is at variance with the above proposals. A possible endogenous mechanism will be outlined in the general discussion.

METHODS

SAND CULTURE: Seeds of the Improved Pearson variety of tomato (*Lycopersicon esculentum* Mill.) were planted in 8-liter glazed crocks containing pure quartz sand and grown in the greenhouse until they had true leaves about three centimeters long. During this period they were watered daily with nutrient solution no. 2 of Hoagland and Arnon (19). A chelated iron compound (sodium ferric diethylenetriamine pentaacetate) was substituted for iron tartrate and used at the concentration of 5 ppm metallic iron. This solution shall be referred to as Hoagland's solution, and dilutions or concentrations thereof as 0.2, 0.4, and 2.0 Hoagland's solution.

Air temperature in the greenhouse fluctuated from a constant night temperature of 18° C to a daily maximum of 35° C. Noon light intensity was about 5,000 ft-c. Daylengths varied from 11.5 to 13 hours. Cloudy days were not encountered. At the start of the treatments, the crocks were placed in water baths which were gradually brought to the desired temperatures. Gravity drainage was provided for the crocks.

SOLUTION CULTURE: Seeds were germinated between sheets of blotting paper tilted to allow seedlings to grow upright. Upon sprouting, the plants were transferred to individual compartments of a single tank. This procedure provided a common nutrient medium, yet prevented root intertwining, and produced uniform plants for experimental use.

When the sixth leaf of such a plant was about three centimeters long, the plant was transferred to treatment conditions. In treatment, each plant constituted an experimental plot. The root medium for each was an aerated solution in an individual glass jar from which light was excluded. These jars in turn were immersed in thermostatically controlled water baths. The plant shoots protruded through corked openings in the water bath covers. By this technique the micro-climate around the shoot was kept independent of the root medium temperature. Treatments were replicated from 6 to 12 times.

The aerial portions of the plants were enclosed under a single plastic-covered frame, through which a constant air movement was maintained. The plastic material permitted a light intensity of approximately 3,500 ft-c at noon. In experiments involving humidity as a variable, this frame was divided by a glass partition, providing two chambers for humidity treatments which transected root temperature treatments. The relative humidity at night approximated 70 % for all plants. Each morning, during experiments which involved humidity, a differential of 15 % in relative humidity was rapidly established between the two chambers, and maintained until sunset. The relatively dry air of the greenhouse was blown unaltered into one chamber. Into the other was blown air which was drawn through moistened filter pads. Air movement was extremely gentle in all cases. By careful adjustment of the ventilation, the difference in humidity could be maintained while providing a natural cycling of illumination and air temperature which was uniform for all plants. Relative humidities were measured with an aspirator-type psychrometer. In experiment IV, relative humidities reached daily minima of about 40 % and 55 %, respectively, for the low and high humidity groups. In experiment VI, the corresponding minima were 35 % and 50 %.

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[2] Represents a portion of a doctoral dissertation.

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CHEMICAL ANALYSES: Shoots and roots used for analysis were dried at 71° C in a forced-draft oven. The dried tissue was ground to pass a 40-mesh screen of a Wiley mill. Total phosphorus was determined by the method of Fiske and Subbarow (15), and cations by flame photometry (6). Nitrate and soluble phosphorus were determined from a 2% acetic acid extract of dried material as outlined by Johnson and Ulrich (22). Nitrate, calculated on a nitrogen basis, is referred to as nitrate-nitrogen (NO_3 as N). In experiments III and V, potassium was determined from the acetic acid extract. Analysis of the residue showed potassium to be completely displaced by this method. In experiment IV (table II & III), the phosphorus fraction designated as insoluble phosphorus refers to the difference between total phosphorus and the acetic acid-soluble fraction.

All analyses, performed in duplicate, are reported on the dry weight basis. Unless otherwise qualified, all treatment differences cited in the text are significant at the 95% or 99% level.

EXPERIMENTAL RESULTS

EXPERIMENT I. *Interaction of nutrition & root temperature*: In an earlier sand culture experiment (29), warm-rooted tomato plants responded more to an increased level of phosphorus than did cool-rooted plants. At all root temperatures, increased phosphorus in the root medium induced higher levels of phosphorus in the shoots. Thus, the phosphorus level in the shoots of cool-rooted plants did not appear to be the factor limiting their growth. It was decided to modify this experiment by varying the nutrient solution as a whole. Plants were grown in sand at three levels of nutrition, 0.2, 0.4, and 1.0 Hoagland's solution, and at two root temperatures, 18° and 24° C.

Plants were thinned uniformly to three per crock, and treatments were replicated four times. The experimental period was 7 days. Tissue was analyzed for phosphorus and potassium.

The interaction mentioned above was observed to reoccur. Plants growing at a root temperature of 18° C did not respond to increased nutrient levels. At 24° C they did. Plants growing in 1.0 Hoagland's solution were 30% greater in dry weight than those in 0.4 Hoagland's solution, and 60% greater than those in 0.2 Hoagland's solution. At a root temperature of 18° C, increased nutrient concentrations raised the shoot concentrations of phosphorus from 0.22% to 0.33%, and of potassium from 2.65% to 4.45%. At 24° C, intensified nutrition increased the concentration of phosphorus from 0.43% to 0.47%, and of potassium from 4.25% to 6.10%.

At the lower root temperature, the growth of the shoot was independent of its nutrient status, at least with respect to phosphorus and potassium.

EXPERIMENT II: *Xylem exudate*: Should root temperature-induced variations in shoot mineral concentrations be due to altered rates of mineral transport, and if the mineral content of the xylem exudate reflects the transport pattern of the intact plant, then the mineral composition of the exudate and of the intact shoot should vary similarly in response to root temperature. The rate of exudation in the tomato, as affected by root temperature, has been studied by Kramer (24), but he did not report on ion concentrations in the exudate. We studied this effect by collecting the 12 hour day or night exudate of decapitated plants growing in sand, watered with Hoagland's solution, at root temperatures of 15°, 21°, and 27° C. The plants were about 20 cm high when topped. The volume of exudate varied as reported by Kramer (24). The exudate was analyzed directly for potassium, phosphorus, and nitrate.

Phosphorus concentration varied from 33 ppm at 15° to 52 ppm at 27° C. Conversely, the concentration of potassium declined from 534 ppm at 15° to 457 ppm at 27°. Nitrate-nitrogen did not vary significantly and averaged 270 ppm.

Because of the divergent nature of these variations, they cannot be attributed to variation in volume flow of exudate. Under like conditions, the concentrations of these elements in the intact shoot invariably increase in response to an increase in root temperature. Just as classical studies have shown poor correlation between salt uptake and water uptake (25), so these data indicate that patterns of salt accumulation in the shoot do not always bear a close relation to salt concentration in the xylem exudate. In addition, note that the concentrations of phosphorus, potassium, and nitrate in tomato exudate are only one to two times that of Hoagland's solution, whereas their concentrations in the intact fresh shoot are from 10 to 40 times their concentrations in Hoagland's solution. The importance of the shoot cells in the concentration phenomenon is treated more fully in the Discussion.

EXPERIMENT III. *24-Hour response*: In the young tomato plant, response of the shoots to a decreased root temperature is discernible within 24 hours. The response is evidenced by a darkening of leaf color and accumulation of purple pigmentation in the stem. If differences in mineral composition are detectable within 24 hours, they should parallel differences found in longer term experiments, provided that an unqualified change in basic rate of transport is responsible.

To determine the 24-hour response, young plants were placed in aerated Hoagland solution held at 13° or 25° C, having developed in Hoagland's solution first at 13°, then at 25° root temperature, in their pre-treatment. Experimental procedures were as previously described. Potassium, nitrate, and phosphorus determinations were made on acetic acid extracts of each individual plant, of which there were 24.

No differences in concentrations of potassium or soluble phosphorus in the shoot, due to treatment, were detectable in 24 hours. Nitrate nitrogen, however, varied from 880 ppm at 13° to 1,060 ppm at

25° C. Average increase in plant weight was 90 mg at 13° and 560 mg at 25° C.

Discussion of Experiments I, II, III: The evidence of the three experiments just described indicates that rate of nutrient supply is not the factor which limits shoot growth at cool root temperatures. The results of experiment I show independence of shoot growth from its nutrient status at the low root temperature. Experiment II discloses the fact that the pattern of mineral accumulation displays considerable independence from the composition of the xylem exudate. The results of experiment III suggest that the reduced amounts of phosphorus and potassium in shoots of cool-rooted plants do not necessarily represent an immediately reduced transport of these ions to the shoot.

The data do not support the idea that a slow rate of nitrate reduction at low root temperature might be a chief factor in the depression of shoot growth. An increase in tissue nitrate would be expected to result from an inhibition of nitrate reduction (12). However, in response to low root temperature, the nitrate content of shoots and roots decreases in both long and short-term trials, and nitrate concentration in the exudate does not vary.

Experiment IV. *Interaction of humidity and root temperature*: Previous to experiment IV, we used two widely differing methods of determining the importance of water stress in the over-all response of tomato plants to a cool root temperature.

The gradual decrease in the moisture content of plant tissues which occurs during the day is well known (25). Our measurements, using root temperatures of 18° and 24° C, failed to detect a greater diurnal moisture loss from the tissues of plants growing in the cooler root media. These measurements were made under many varied conditions of pretreatment and nutrient media. A 12-hour nocturnal period of adjustment to root temperature was allowed before measurements were started.

Eaton (11) demonstrated that a tomato plant having a portion of its root system in a nutrient solution will remove water amply from a distilled water supply via the remaining portion of its root system. Working on this basis, we grew plants with part of the root system in a warm or cool nutrient solution and the other part in warm distilled water. Several types of control treatments were provided. Results showed that the growth of plants in cool root media was not enhanced by provision of an auxiliary warm water supply.

In experiment IV, the approach to this problem was to superimpose humidity treatments (15 % R.H. differential) on plants growing at two root temperatures (15° & 25° C) in solution culture. The reasons for using a narrow humidity differential are discussed later. Under these conditions it was expected that shoot growth would be enhanced by higher humidity if water stress was a limiting factor. In addition, the use of 1.0 and 2.0 Hoagland's solution was introduced since such a variation is known to affect water absorption, and to expand our body of data on the interaction of root temperature and nutrient concentration. The treatment period was 7 days.

The effects of root temperature, humidity, and root medium concentration on shoot and root growth are shown in table I. Considering, first, only plants growing in 1.0 Hoagland's solution, the standard treatment, it is evident that higher humidity signifi-

Table I

Effects of Root Temperature, Relative Humidity, & Nutrient Concentration on Shoot & Root Growth*

Nutrient conc	Root temp	Per shoot				Per root				Shoot-root ratio***	
		Fr wt		Dry wt		Fr wt		Dry wt			
		Rel hum., %		Rel hum., %		Rel hum., %		Rel hum., %			
standard	° C	40	55	40	55	40	55	40	55	40	55
		grams								g/g	
1.0	15	5.1	5.7	0.40	0.44	1.6	1.4	0.10	0.10	4.1	4.6
	25	8.1	10.1	0.56	0.70	1.9	2.2	0.09	0.10	6.5	6.9
2.0	15	4.4	5.3	0.35	0.43	1.3	1.5	0.08	0.10	4.6	4.4
	25	7.3	7.3	0.53	0.51	1.7	1.7	0.10	0.10	5.6	5.5
Significant levels of mean difference, %											
Root temperature		99		99		99		N.S.		99	
Nutrient conc.		99		99		95		N.S.		97.5	
Relative humidity		97.5		97.5		N.S.		N.S.		N.S.	
Interaction**		95(H×T×C)		95(H×T×C)		N.S.		N.S.		99(T×C)	

* Experiment IV.

** All possible interactions are nonsignificant except those which are shown.

*** Dry weight basis.

T, root temperature; C, nutrient concentration; H, relative humidity.

TABLE II

EFFECTS OF ROOT TEMPERATURE, RELATIVE HUMIDITY, & NUTRIENT CONCENTRATION ON MINERAL COMPOSITION OF SHOOT*

NUTRIENT CONC	ROOT TEMP	K		SOL. P***		INSOL. P***		Ca		NO_3 AS N	
		REL. HUM., %		REL. HUM., %		REL. HUM., %		REL. HUM., %		REL. HUM., %	
STANDARD	° C	40	55	40	55	40	55	40	55	40	55
		g/100 g *Dry weight*									
1.0	15	4.8	4.8	0.31	0.33	0.25	0.24	3.2	3.2	0.79	0.85
	25	6.1	5.7	0.54	0.55	0.25	0.25	3.2	3.2	1.04	1.16
2.0	15	5.0	5.0	0.37	0.36	0.23	0.25	3.3	3.1	0.88	0.90
	25	5.7	5.6	0.52	0.54	0.26	0.25	3.3	3.1	1.19	1.17
Significant levels of mean difference, %											
Root temperature		99		99		N.S.		N.S.		99	
Nutrient conc.		N.S.		92		N.S.		N.S.		N.S.	
Relative humidity		N.S.		92		N.S.		N.S.		97.5	
Interaction**		95(T×C)		99(T×C)		N.S.		N.S.		97.5(H×C)	

* Experiment IV.

** All possible interactions are nonsignificant except those which are shown.

*** Acetic acid soluble or insoluble phosphorus.

cantly promoted shoot growth in warm-rooted plants, but did not do so in cool-rooted plants. A separate statistical analysis of this data (1.0 Hoagland's solution) showed differences due to root temperature or humidity to be highly significant as was the interaction of root temperature and humidity. Root growth exhibited a positive response to root temperature if measured by fresh weight, but not by dry weight. The shoot-root ratios indicate that the warmer root temperature stimulated shoot growth more than root growth.

The more concentrated nutrient solution consistently depressed shoot and root growth, and at the warm root temperature depressed shoot growth more than root growth (see shoot-root ratios). The depressing effect of concentration was not additive to that of cool root temperature. The 2.0 Hoagland's solution induced the greatest growth depression when the root temperature was warm and the humidity relatively high (i.e., when growth was normally the fastest). This suggests that cool root temperature

TABLE III

EFFECTS OF ROOT TEMPERATURE, RELATIVE HUMIDITY, & NUTRIENT CONCENTRATION ON MINERAL COMPOSITION OF ROOT*

NUTRIENT CONC	ROOT TEMP	K		SOL. P***		INSOL. P***		Ca		NO_3 AS N	
		REL. HUM., %		REL. HUM., %		REL. HUM., %		REL. HUM., %		REL. HUM., %	
STANDARD	° C	40	55	40	55	40	55	40	55	40	55
		g/100g *Dry weight*									
1.0	15	7.0	6.6	0.63	0.55	0.64	0.64	0.75	0.63	1.18	1.08
	25	7.5	7.8	0.81	0.74	0.62	0.57	0.96	0.67	1.38	1.36
2.0	15	6.8	7.7	0.80	0.70	0.74	0.65	0.82	0.75	1.27	1.24
	25	7.4	7.9	0.84	0.85	0.54	0.52	0.96	0.91	1.53	1.50
Significant levels of mean difference, %											
Root temperature		99		99		99		99		99	
Nutrient conc.		N.S.		99		N.S.		97.5		99	
Relative humidity		97.5		99		N.S.		99		N.S.	
Interaction**		99(H×T)		95(H×T)		94(T×C)		92(H×C)		N.S.	
		95(T×C)		99(T×C)							
		97.5(H×T×C)		92(H×T×C)							

* Experiment IV.

** All possible interactions are nonsignificant except those which are shown.

*** Acetic acid soluble and insoluble phosphorus.

and nutrient concentration were not active through the same mechanism.

It will be noticed (table II) that an increase in humidity did not greatly affect the concentrations of mineral elements in the shoot. Doubling the standard concentration of Hoagland's solution had the same general lack of effect, depending on levels of root temperature and humidity. Environmental effects on mineral accumulation in the roots were complex (table III).

Nightingale and Mitchell (34) found that tomato plants grew more vigorously in response to increased humidity. Freeland (16) found the opposite to be the case. In our preliminary experiments, shoot growth was significantly reduced by a very high humidity (90 %) when maximum daily light intensity was about 1,500 ft-c. Clearly, the effect of humidity on shoot growth depends on several qualifying circumstances. The conditions of experiment IV are considered valid for the primary purpose of the investigation. The range in intensities of the variables was narrow and closely approximated greenhouse conditions. An abnormal situation was not created. The humidity levels employed appear to be in the limiting region where a small variation would be expected to evoke a tangible response in a dependent variable. From the results of our experiments it may be concluded that water stress is not the factor responsible for the shoot growth depression occurring at cool root temperatures.

EXPERIMENTS V & VI: With the indication that rates of mineral and water absorption are causally unimportant in the relationship of shoot growth to root temperature, the possibility of an endogenous mechanism remained to be explored. By growing plants in dilute salt solutions and distilled water, it was speculated that the situation might occur in which no net movement of salts to the shoot would take place in response to root temperature treatment. Should this occur, the response or lack of response of shoot growth to root temperature would have particular significance.

Furthermore, it was possible in experiments I and IV that an actual limiting role exerted by water or ion transport was masked because both were limiting or nearly so in plants growing in cool root media. If such were the case, the limiting role would be interchanged in experiments testing each separately, and the limiting nature of neither would be disclosed. To test this possibility, plants growing in distilled water were exposed to two different levels of humidity. With one factor thus definitely limited, the apparency of shoot response to variation in the other could be ascertained.

TABLE IV

COMPOSITION OF SOLUTIONS IN EXPERIMENT V

SOLUTION	MOLARITY	K	P	N
		ppm (*approx*)		
KCl	0.0001	4	...	...
KH_2PO_4	0.0001	4	3	...
NaH_2PO_4	0.0001	...	3	...
0.01 Hoagland (macro only)		2.3	0.3	2
1.0 Hoagland (for reference)		234	32	200

EXPERIMENT V. *Response to root temperature of plants growing in dilute solutions*: Plants previously grown in 1.0 Hoagland's solution, 15° C in tem-

TABLE V

INFLUENCE OF VARIOUS DILUTE SALT SOLUTIONS & ROOT TEMPERATURE UPON RATE OF PLANT GROWTH & SHOOT-ROOT RATIO*

	% FR WT INCREASE**		% DRY WT SHOOT		SHOOT-ROOT RATIO***	
ROOT TEMP (C):	15°	25°	15°	25°	15°	25°
Solution						
KCl	37	46	12.8	12.9	2.72	2.40
KH_2PO_4	37	44	13.7	13.0	2.71	2.53
NaH_2PO_4	40	46	13.5	13.0	2.45	2.70
0.01 Hoagland	45	52	13.3	13.4	2.18	2.31
Significant levels of mean diff., %						
Root temperature	99		N.S.		N.S.	
Solution	94		N.S.		N.S.	

* Experiment V.

** Gain in fresh weight as a per cent of the initial fresh weight.

*** Fresh weight basis.

perature, were placed in 0.25 Hoagland's solution 5 days before beginning treatment. In treatment, they were grown at 15° or 25° root temperature, and in one of the four nutrient solutions whose characteristics are shown in table IV. Also shown, for reference, are some properties of the Hoagland's solution. One-half the plants were taken for weight measurement at the end of 48 hours. The nature of their responses was identical to that of the remaining plants, which were taken for weighing and analysis after an additional 72 hours. Plants were composited within treatments for chemical analysis.

Plants grew proportionately faster in the warm than in the cool dilute solutions, as shown by the data on per cent increase (table V). Since temperature treatment caused no shift in shoot-root ratios, it may be assumed that warm root media promoted shoot and root growth to the same degree.

Nitrate reserves were nearly depleted in all plants, particularly in faster growing warm-rooted plants, being about 100 ppm in shoots and 400 ppm in roots on a dry weight basis. The influence of treatment on concentrations of potassium and acetic acid-soluble phosphorus are shown in table VI. Shoots of warm-rooted plants were invariably higher in potassium and soluble phosphorus than their cool-rooted counterparts, even when these elements were not present in the nutrient solution. Evidently the warm root temperature stimulated the accumulation potential of shoot cells preferentially over that of root cells. In all solutions, the concentration of potassium in the root declined at the warmer root temperature. With phosphorus this occurred only when there was no phosphate in the nutrient medium.

Table VI also presents the absolute amounts of potassium and acetic acid-soluble phosphorus in whole plants, corrected for initial differences in plant weights. Warm-rooted plants contained more of these elements, even when they were not supplied in the external solution. This suggests that they were lost preferentially to the cooler solution. The differences amounted to 8 % of the potassium and 17 % of the soluble phosphorus. To what extent the soluble phosphorus fraction is indicative of total phosphorus is of course subject to doubt.

Experiment VI. *Response to root temperature of plants growing in distilled water*: Plants were preconditioned in a manner designed to make them typical of neither warm-root nor cool-root plants at time zero. During the final pre-treatment phase they were grown in standard Hoagland's solution at 25° for 3 days. During treatment, plants were grown in aerated distilled water, 15° or 25° C in temperature, and either at 35 % or 50 % minimal relative humidity. After 68 hours at treatment, plants were weighed and combined in groups of two for chemical analysis.

A slight response in shoot growth to root temperature was observed (table VII). Two preliminary experiments resulted in responses of the same level of significance (90–92 %). Final shoot weights as a per cent of the plants' original weights take initial differences into account. Such corrected quantities may more precisely portray the true responses, since growth was slow in distilled water and the treatment period was brief. The probability is fairly high that higher root temperature and higher humidity enhanced shoot growth even under these conditions of severe nutrient limitation. The shoot-root ratios indicate that the warm root temperature promoted shoot growth less than root growth.

In general, plants attaining a greater rate of growth, due to treatment, had lower concentrations of potassium, phosphorus, calcium, and nitrate in both root and shoot (table VIII). As estimated by several quantities or ratios abstracted from the data, on an absolute basis, differential shoot-root shifts in phosphorus and potassium, due to treatment, were too small to be detectable, nor were they lost to the medium in greater amounts at one temperature than at another. In contrast, calcium was lost from the shoot preferentially at the warm root temperature, and to a greater degree from the root, with the result that warm-rooted plants significantly contained from 5 % to 10 % less calcium than cool-rooted plants.

Table VI

Influence of Various Dilute Salt Solutions & Root Temperature on Concentration & Absolute Amounts of Potassium & Phosphorus**

Root temp, C:	g K/100 g Dry wt				K/Plant***		g Sol. P/100 g dry wt				Sol. P/Plant***	
	15°		25°		15°	25°	15°		25°		15°	25°
Solution	Shoot	Root	Shoot	Root	mg/g		Shoot	Root	Shoot	Root	mg/g	
KCl	3.4	3.8	3.5	2.8	5.1	5.3	0.14	0.27	0.16	0.24	0.23	0.27
KH_2PO_4	3.0	3.6	3.2	3.1	4.8	5.0	0.21	0.38	0.28	0.57	0.36	0.50
NaH_2PO_4	2.8	3.1	3.2	2.6	4.5	4.9	0.20	0.41	0.27	0.46	0.36	0.47
0.01 Standard	2.9	3.5	3.4	2.6	4.7	5.4	0.15	0.30	0.17	0.21	0.27	0.29

* Experiment V.

** Acetic acid soluble phosphorus.

*** Milligrams element per plant divided by the initial fresh weight per plant.

TABLE VII

EFFECT OF ROOT TEMPERATURE & RELATIVE HUMIDITY ON GROWTH OF SHOOTS & ROOTS OF PLANTS GROWING IN DISTILLED WATER*

REL. HUM.	ROOT TEMP	PER 2 SHOOTS			PER 2 ROOTS		SHOOT-ROOT RATIO***
		FR WT	ADJ. FR WT**	DW/FW	FR WT	DW/FW	
%	°C	g	%	%	g	%	
35	15	5.7	96	11.2	2.0	5.4	6.0
	25	6.2	96	11.6	3.4	5.7	3.8
50	15	6.4	98	11.6	2.2	5.5	6.5
	25	6.5	102	11.5	3.3	5.5	4.2
Significant levels of mean diff., %							
Root temp		92	92	N.S.	99	N.S.	99
Rel. hum.		92	97.5	N.S.	N.S.	N.S.	N.S.
Interaction		95	90	N.S.	N.S.	N.S.	N.S.

* Experiment VI.
** Fresh weight of shoot as a per cent of the initial fresh weight of the entire plant.
*** Dry weight basis.

DISCUSSION OF EXPERIMENTS V & VI: The fact that increased humidity (experiment VI) promoted shoot growth when nutrition was severely limited makes it improbable that water and nutritional relations exerted an interchangeable limiting role in root temperature responses. This substantiates the results of experiments I and IV.

In experiment VI, shoots responded slightly to warm distilled water, although concomitantly experiencing a net loss in nitrate and calcium, and without gaining phosphorus and potassium. Apparently the initial stimulation was due to some factor other than mineral supply. The stimulus, in this case, was not sustained by an adequate supply of mineral ions, and shoot growth was not vigorous. The relationship of the stimulation and the mineral supply is demonstrated by these observations on shoot-root ratios: in 1.0 Hoagland's solution (expt. IV), shoot growth was stimulated more than root growth by warm root temperature; in extremely dilute solutions (expt. V), shoot and root growth were stimulated equally; in distilled water, warm root temperature stimulated root growth more than shoot growth.

In experiment V, phosphorus and potassium apparently were lost to the medium in greater amounts at the cooler root temperature. In experiment VI, calcium was lost in greater amounts to the warmer root medium. These directional variations compare favorably with results obtained under conditions of normal nutrition; i.e., it was originally reported by

TABLE VIII

EFFECT OF ROOT TEMPERATURE & RELATIVE HUMIDITY ON CONCENTRATION OF POTASSIUM, PHOSPHORUS, CALCIUM, & NITRATE IN SHOOTS & ROOTS OF PLANTS GROWING IN DISTILLED WATER*

REL. HUM.	ROOT TEMP	K		SOL. P		TOTAL P		Ca		NO_3 AS N	
		SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT
	°C	*% Dry Weight*								ppm	ppm
35	15	3.3	4.2	0.20	**	0.36	0.67	1.8	0.97	930	1700
	25	3.2	2.8	0.20	**	0.35	0.41	1.7	0.47	330	500
50	15	3.1	4.2	0.18	**	0.34	0.64	1.7	0.84	780	1700
	25	2.9	2.6	0.19	**	0.34	0.42	1.6	0.47	340	400
Significant levels of mean difference, %											
Root temperature		N.S.	99	97	...	N.S.	99	99	99	99	99
Relative humidity		95	N.S.	95	...	N.S.	N.S.	97	N.S.	N.S.	N.S.
Interaction		N.S.	N.S.	99	...	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

* Experiment VI.
** Not analyzed.

Lingle and Davis (29) that cooler rooted plants contained less potassium and phosphorus, while the warmest rooted plants contained less calcium. It is possible that the quantitative effect of ion loss to the medium upon the mineral content of the shoot is not negligible, even when nutrition is normal.

One additional observation upon intra-plant movements of minerals deserves comment. In experiment V, the shift of phosphorus and potassium from root to shoot was greater at warmer root temperature. The apparent loss of phosphorus and potassium to the medium was greater at cooler root temperature. This may indicate an effective barrier between the free space and a portion of the root-shoot path. The exudation phenomenon must, of course, rest upon just such a basis (8, 20). Its importance in the overall transport scheme is being evaluated at present (26, 37, 40). (It is emphasized that the above-mentioned differential movements of phosphorus and potassium were not evident in the distilled water experiment.) Ion loss to the medium and ion release to the shoot may involve fundamentally different mechanisms, even structures.

Discussion

If shoot response to root temperature has a degree of independence from its nutritional and hydrational states, an endogenous mechanism must be proposed. Variations in root temperature may, for instance, induce differential production of root-produced substances having shoot regulatory activity. Evidence of such substances has been reported for tomato (21, 42). Consideration of this type of mechanism probably will not be neglected in future investigations. Another mechanism is possible, however. It is easily overlooked, and, for this reason, will be emphasized in this discussion. Whenever shoot-root relationships are being studied, this mechanism remains a possibility until discounted. This is particularly true when experimental procedures involve ringing, or girdling, or severing of the root from the shoot.

Relatively low temperature may retard the rate at which materials are transported in the phloem (14). It would be reasonable to suspect that low root temperature might diminish the root's effectiveness as a "sink" for phloem transported material (7). Indeed, cool root temperature decreases shoot growth and mineral accumulation in a manner which recalls the ringing of branches (9, 31). Similar results have been produced by cooling a portion of a branch (10). In cooling and ringing treatments, procedures were used in which root activities were not curtailed nor transpiration primarily affected. Results of ringing were interpreted as indicating a significant upward transport of mineral elements in the phloem—a widely contested conclusion.

A different interpretation is possible. In the normal functioning of shoot cells, a large number of compounds are produced of which many may be classified as growth substances (17). Several known growth substances have been shown to accumulate at girdles (5). Should the shoot experience an alteration in the concentration of such compounds, due to an interruption or depression of phloem transport, a partial inhibition of shoot cell metabolism might result.

Endogenous mechanisms have been shown to cause wide fluctuations in the intensity with which cells accumulate mineral ions (3, 28, 32, 43). Such accumulation is known to depend on metabolic activity (1, 18, 37, 41). When shoot activity is comparatively low, mineral elements which have been taken up and not accumulated are probably subject to phloem export, or, in girdled stems, to a backward diffusion along purely physical gradients within the free space of the plant body (26).

Many of the responses reported herein, with regard to mineral concentrations in the shoot, are difficult to account for on the basis of a unidirectional (upward) transport (20, 40) alone. The mineral composition of the xylem exudate and that of the shoot did not vary similarly in response to root temperature, nor did the shoot display similar long and short-term variations. Doubling the standard Hoagland's solution did not increase the salt content of shoots, nor did elevated humidity decrease it, contrary to prediction on the basis of passive transport. Losses of ions from the shoot and from the entire plant, presumably to the rooting medium, are to be inferred from analyses of tissues of plants which were grown in dilute salts and distilled water.

These observations are more consistently explained if the mineral content of the shoot is assumed to be at least partially attributable to a selective accumulation by the shoot cells (20). Penston (36), Alberda (1), and Helder (18) propose a return of excess ions to the roots and to the root medium. Recirculation of ions has been thoroughly demonstrated (4). Mineral excretion to the root medium has been extensively reviewed by Loehwing (30) and Helder (18), and more recently demonstrated by Kylin (27) and Emmert (13), among others. Quantification of such mineral retranslocations may be essential in a strict accounting of the shoot's mineral content at a given time.

Should cool root temperatures actually retard movement in the phloem, resulting in a congestion of substances in the shoot which can depress metabolic activity and salt accumulation, a basis may be provided for understanding some of the effects of cool root temperature on shoot growth and mineral accumulation in tomato plants.

Summary

Tomato plants were grown under partially controlled environmental conditions in nutrient culture, the main treatments being variations of root temperature in the range 13° to 27° C.

In nutrient solutions graded in concentration from

0.2 to 1.0 times the standard strength of Hoagland's solution, each increment in nutritional level enhanced shoot growth of warm-rooted plants, but not of cool-rooted plants, although phosphorus and potassium concentrations were increased in both warm and cool-rooted plants. An increase of atmospheric humidity promoted the shoot growth of warm-rooted but not of cool-rooted plants. When plants were growing in distilled water, a weak but significant promotion of shoot growth was observed in response to increased root temperature and increased humidity. In this situation, the faster shoot growth occurred without a more rapid net movement of phosphorus and potassium into the shoot, and despite an absolute decrease in nitrate and calcium. It was concluded that the control of shoot growth by root temperature does not reside primarily in rates of mineral or water supply to the shoot. Over-all evidence did not favor an important role for nitrate reduction in this connection.

In response to root temperature, variations in mineral contents of the xylem exudate were not similar to variations in mineral contents of intact shoots, nor were short-term responses similar to long-term responses. A nutrient medium concentration twice that of Hoagland's solution did not materially alter the salt status of the shoots, nor did moderately increased humidity. Observations made on plants grown in dilute salt solutions and distilled water demonstrated the necessity of considering ion movements out of the plant in accurately accounting for its mineral contents at a given time. It was concluded that the salt status of shoots in response to root temperature variations could not be accounted for entirely by estimations of uptake alone, but shoot export and plant loss of elements must also be considered.

In explaining the response of shoot growth and salt accumulation to root temperature, discussion centers upon a possible endogenous mechanism.

Acknowledgments

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NUCLEOTIDE & RIBONUCLEIC ACID METABOLISM OF CORN SEEDLINGS[1]

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The consideration of nucleotides and ribonucleic acid (RNA)[2] in metabolism has become increasingly important in cellular biology during the past few years. The commonly occurring nucleotides of wheat and corn plants have been identified by Bergkvist (1) and Cherry and Hageman (3), respectively. The latter have shown that the nucleoside di- and triphosphates in etiolated corn embryos shift to the nucleoside monophosphates as a function of growth.

Little is known about the metabolism of nucleic acid during seed germination. Oota et al (11, 12) have examined nucleic acid metabolism in germinating seeds, and have found large amounts of RNA stored in the cotyledons of a bean. Germination resulted in a steady decrease in the RNA content of the cotyledons with an increased RNA content in the seedling. The presence of the embryo was essential for breakdown of RNA in the cotyledons. If embryos were detached from the cotyledons, no RNA degradation occurred (13).

Recently, Cherry et al (4) have shown that RNA is synthesized in etiolated corn embryonic axis but degraded in the scutellum with both reactions being greater in hybrid plants than in their inbred parents.

Oota and Takota (12) have proposed that there are at least two types of RNA-s i.e. functional and transportable, in bean germ tissues. Their results appear to illustrate conversion steps from functional RNA to transportable RNA.

Hanson (6) indicates that RNA in membranes is implicated in ion accumulation, solute retention, and oxidative phosphorylation.

The experiments reported here represent studies on the metabolism of nucleotides, RNA, and protein in various cytoplasmic fractions of corn seedlings. Incorporation of P^{32} into various soluble nucleotides and cytoplasmic RNA-s, and ADP-C_8^{14} uptake by excised tissue were studied.

MATERIALS & METHODS

PLANT MATERIAL: Hybrid corn seed[3] (*Zea mays* L. var. WF9XM14) were lightly dusted with a fungicide (Spergon, U. S. Rubber Co.) and approximately 100 were placed embryo down on paper towelling supported by a layer of gravel (400 ml) in a 2 quart pyrex utility dish. The gravel and towelling were saturated with 200 ml of deionized water containing 0.5 mc of P^{32} (5.2 μg) as potassium phosphate. The seeds were germinated in darkness in a humid atmosphere at 29° C for 1, 2, 3, or 4 days. Additional deionized water was added to the dish during the germination period to keep the gravel saturated. Seedlings were harvested and washed several times with deionized water to remove excess P^{32} before use.

Plant material for the ADP-C_8^{14} studies was obtained by the above procedure except that the seeds were moistened with 10^{-4} M $CaCl_2$ instead of the radioactive phosphate solution.

ISOLATION OF CYTOPLASMIC PARTICULATES: The plant tissue (about one gm) was homogenized for 3 minutes in 5 ml of deionized water with an ice-jacketed glass homogenizer with a power-driven Teflon pestle. The homogenate was cleared of cellular debris by centrifugation at 500 *g* for 5 minutes. An aliquot of the debris-free homogenate was subjected to differential centrifugation in a Spinco model L ultracentrifuge, each successive particulate fraction being obtained from the supernate of the preceding fraction. The centrifugal forces, time, and the arbitrary designation of the isolated fractions were as follows: mitochondria, 22,000 *g* for 15 minutes; microsomes, 110,000 *g* for 1 hour; cytoplasmic sap, particulate-free supernate. The terms used to designate the cytoplasmic fractions are operational, as listed above, and do not necessarily mean the presence of pure morphological entities.

An isotonic grinding medium (0.5 M sucrose) was also used in the isolation of particulate fractions. Analysis of the particulates obtained from water and 0.5 M sucrose extractions were comparable; therefore, only the results from water extraction will be reported.

[1] Received August 5, 1960.

[2] The following abbreviations will be used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; GTP, guanosine triphosphate; CMP, cytosine monophosphate; CTP, cytosine triphosphate; DPN, diphosphopyridine nucleotide; TCA, trichloroacetic acid; RNase, ribonuclease; and RNA, ribonucleic acid.

[3] We are indebted to Dr. W. J. Mumm, Crow's Hybrid Corn Co., Milford, Ill., for the corn seed used in this work.

ANALYSIS OF CYTOPLASMIC FRACTIONS: The total acid-soluble nucleotides and RNA were extracted and measured by a method (5) similar to that of Ogur and Rosen (10). Aliquots of each of the water suspended particulate fractions (mitochondrial & microsomal) and the cytoplasmic fraction, and debris-free homogenate were acidified with 1.2 M $HClO_4$ to a final molarity of 0.2 which precipitated the RNA. The RNA precipitates were isolated by centrifugation and washed twice with 0.2 M $HClO_4$. The entire extraction procedure for the acid soluble nucleotides was performed at 2° C. The supernatant solution and the two washes were combined, made to volume, and the soluble nucleotides estimated by the difference of absorbancy at 260 and 290 mμ using AMP as a standard.

The precipitated RNA pellets were extracted thrice with 2:2:1 ethanol-ether-chloroform to remove lipids. RNA was hydrolyzed by incubating the lipid-free residue with 0.5 M $HClO_4$ at 70° C for 40 minutes. Absorbancy differences at 260 and 290 mμ of the cleared supernatant solution were referred to a standard curve obtained from similarly treated yeast RNA.

Protein was determined by the method of Lowry et al (8) on ice-cold 10% TCA precipitates of each of the cytoplasmic fractions.

DETERMINATION OF RADIOACTIVITY: Aliquots of the acid-soluble nucleotides and of the hydrolyzed RNA were neutralized with KOH and the $KClO_4$ was removed by centrifugation. Each of the nucleotide and hydrolyzed RNA samples was dried and the radioactivity counted.

ADP-C_8^{14} UPTAKE: Root tips, 1 cm long, were sectioned from primary and secondary roots, apical mesocotyl sections (1 cm), and scutella were removed from 3-day-old corn seedlings. The tissue was rinsed in deionized water, blotted, and rapidly weighed to the nearest milligram before use. Tissue samples were placed in petri dishes and incubated in the light at room temperature with 30 ml of a solution containing 1% sucrose, 10^{-3} M KH_2PO_4, and 1 μc of ADP-C_8^{14} (0.8 mg) adjusted to pH 6.3 with NH.OH. After 8 hours the tissue was removed, rinsed several times with deionized water, weighed, and frozen.

Samples of the fresh tissue were frozen for initial nucleotide and RNA determinations.

NUCLEOTIDE EXTRACTION & ION-EXCHANGE CHROMATOGRAPHY: Scutellum and radicle tissues were removed from the etiolated corn seedlings (3 days old) and the acid-soluble nucleotides extracted from each tissue with ice-cold 0.6 M $HClO_4$. The procedures for extraction and column chromatography of the nucleotides were the same as previously described (3), except that Dowex-1 × 8 was used instead of Dowex-1 × 10. The eluate was collected in 10-ml volumes. The nucleotide content was determined by the absorption of light at 260 mμ and 1-ml aliquots from each fraction were dried and counted for radioactivity.

PAPER CO-CHROMATOGRAPHY OF ORGANIC PHOSPHATES & ORTHOPHOSPHATE: Samples obtained by concentrating the eluate of peak fractions from column chromatography were co-chromatographed on paper with standard phosphate compounds in different solvents. The solvents most used for chromatography were *n*-propanol : NH_4OH : H_2O (60:30:10) and isobutyric acid : NH_4OH : H_2O (57:4:39). After the paper chromatograms were developed and dried, phosphorus-containing compounds were detected with a molybdic acid spray reagent (2). In some instances in which the unknown radioactive samples were too dilute to react with molybdic acid, the paper was cut into 2-cm strips and these were in turn cut into 2-cm squares. Each of the squares was counted for radioactivity, thereby establishing the location of the unknown on the paper.

All results reported in this paper are based on two or more separate experiments. Each assay was made in duplicate.

RESULTS

CHANGES DURING SEED GERMINATION: Typical dry weight changes of corn scutellum and radicle are given in table I. The scutellum decreased in dry weight about 18% whereas the radicle increased in dry weight 42-fold in the 3-day period (1–4 days).

As corn seed germinate there is a production of nucleotides in both scutellum and radicle tissues (table II). An interesting facet of RNA metabolism during corn seed germination is that 57% of the scutellum RNA was degraded by the 4th day whereas radicle RNA increased 182%. Growth (dry wt) of the radicle occurred at the same rate as nucleotide synthesis, but RNA was synthesized at a much slower rate.

Protein metabolism was similar to RNA metabolism in the scutellum and radicle tissues; however, the RNA to protein ratio was not constant for either tissue. The RNA to protein ratios (μg/mg) in the scutella were 67, 79, 57, and 51 from 1 to 4 days, inclusively, whereas the ratios in the radicles were 160, 143, 79 and 96.

Changes in protein content in the designated fractions of radicle and scutellum are represented in

TABLE I

DRY WEIGHT CHANGES OF CORN SCUTELLUM & RADICLE TISSUE WITH GROWTH

AGE, DAYS	DRY WT/SCUTELLUM mg	DRY WT/RADICLE mg
1	24.5	0.6
2	23.1	6.8
3	21.0	18.5
4	20.1	26.1

TABLE II

CHANGES IN CYTOPLASMIC NUCLEOTIDES, RNA & PROTEIN IN CORN SEEDLINGS WITH GROWTH

AGE, DAYS	μg/SCUTELLUM			μg/RADICLE		
	NUCLEOTIDES	RNA	PROTEIN	NUCLEOTIDES	RNA	PROTEIN
1	39	150	2,230	9	51	320
2	51	145	1,830	118	96	670
3	80	99	1,720	268	97	1,230
4	80	65	1,290	369	144	1,500

figure 1. The protein content in all the cytoplasmic fractions of the radicle (fig 1–A) increased. However, the major protein increase was in the mitochondrial and cytoplasmic sap fractions with the microsomal protein increasing very little. In general, the cytoplasmic protein in the radicle was almost evenly divided into mitochondrial and cytoplasmic sap proteins.

Scutellum cytoplasmic proteins decreased in all fractions (fig 1–B). The greatest protein loss was from the cytoplasmic sap. The amount of mitochondrial protein remained essentially the same on the 2nd day and decreased on the 3rd and 4th days.

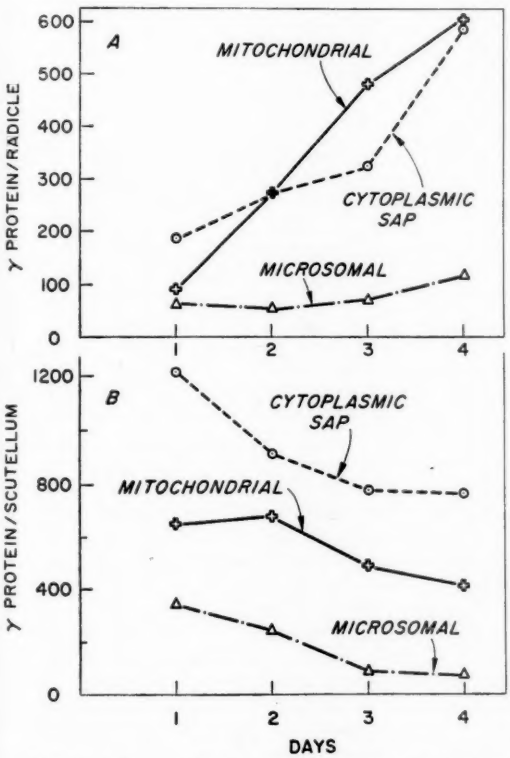


FIG. 1. Changes in the protein content in various cytoplasmic fractions of corn radicles and scutella during seed germination and growth.

Microsomal protein in the scutellum decreased in a linear manner from 1 to 3 days with no essential loss on the 4th day.

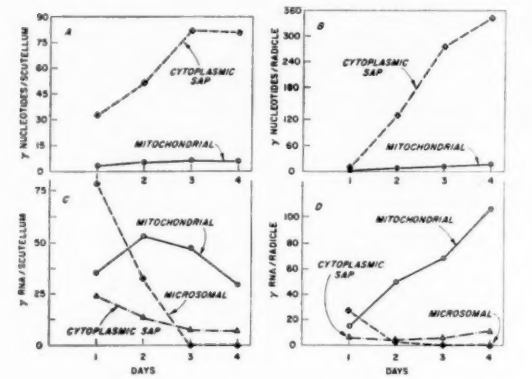
Changes in nucleotide and RNA contents in the cytoplasmic fractions of scutellum and radicle tissues are illustrated in figure 2. No nucleotides were detected from the microsomal fraction with small amounts from the mitochondrial fraction. The scutellum and radicle cytoplasmic sap nucleotides (fig 2–A & 2–B) increased the 2nd and 3rd days at average rates of 24 and 130 μg per day, respectively. There was no change in scutellum nucleotides on the 4th day with the radicle nucleotides increasing slightly.

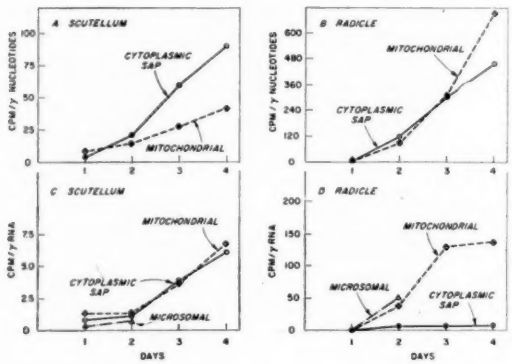
Over 50 % of the RNA content of 1-day old corn scutella was in the microsomes (fig 2–C). However, the greatest RNA degradation in the scutellum occurred in the microsomes with the RNA content decreasing to almost zero on the 3rd day. Mitochondrial RNA increased on the 2nd day (17 μg) and decreased 6 and 18 μg on the 3rd and 4th days, respectively. Cytoplasmic sap RNA of the scutellum decreased at an average rate of 8 μg per day from 1 to 3 days with no change in the RNA content on the 4th day.

About half of the RNA content of 1-day old radicles was in the microsomes with most of the RNA of 2-, 3- and 4-day-old radicles in the mitochondria (fig 2–D). Mitochondrial RNA of the radicle increased from 1 to 4 days at an average rate of 30 μg per day. Microsomal RNA of the radicle decreased to almost zero by the 2nd day with no change thereafter. Cytoplasmic sap RNA remained unchanged on the 2nd and 3rd days with the content slightly increasing on the 4th day.

The relative amounts of radioactive inorganic phosphate incorporated into nucleotides and RNA in the cytoplasmic fraction of scutellum and radicle tissues are illustrated in figure 3. The relative rates of phosphate incorporation into the mitochondrial and cytoplasmic sap nucleotides of the scutellum (fig 3–A) were linear from 1 to 4 days. The cytoplasmic sap nucleotides were labeled with P^{32} at a faster rate and incorporated a larger amount than the mitochondrial nucleotides. The mitochondrial and cytoplasmic sap nucleotides (fig 3–B) of the radicle were labeled at the same rate on the 1st, 2nd, and 3rd days, but the mitochondrial nucleotides were labeled at a faster rate on the 4th day. The rate of phosphate incor-

poration into both the mitochondrial and cytoplasmic sap nucleotides increased with time. No attempt was made to correct for the amount of labeled inorganic phosphate in the acid-soluble (nucleotide) fraction.





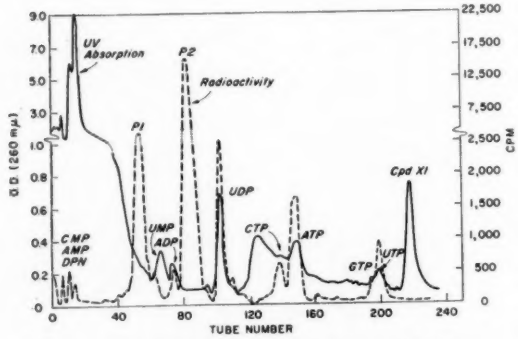


FIG. 2. (*top*). Changes in nucleotide and RNA contents in various cytoplasmic fractions of corn radicles and scutella during seed germination and growth.

FIG. 3. (*center*). The incorporation of radioactive phosphate into cytoplasmic nucleotides and RNA-s during seed germination and growth.

FIG. 4. (*bottom*). An elution-chromatogram of the acid-soluble compounds from 3-day-old corn radicles grown in a radioactive phosphate medium.

No radioactive phosphate was incorporated into the cytoplasmic RNA-s of the scutellum (fig 3-C) on the 1st and 2nd days. The mitochondrial and cytoplasmic sap RNA-s incorporated only small amounts of radioactive phosphate on the 3rd and 4th days. The low amount of phosphate incorporated may be due to exchange reactions rather than de novo synthesis of RNA from nucleosides and inorganic phosphate since mitochondrial and cytoplasmic sap RNA-s in the scutellum are being degraded at the time when small amounts of the label are incorporated.

The relative amount of phosphate incorporated into the microsomal RNA is reported only for 1- and 2-day-old scutella (fig 3-C) and radicles (fig 3-D), because very small amounts or no RNA was detected in the microsomal fractions of older tissue. Even though the amount of RNA in the microsomes of 3- and 4-day-old seedlings was too little to be measured accurately, a fair amount of radioactivity could be measured.

The majority of the radioactive phosphate incorporated into the cytoplasmic RNA-s of the radicle was in the mitochondria (fig 3-D). The rate of phosphate incorporation into the mitochondrial RNA was nearly linear from 1 to 3 days with none being incorporated on the 4th day. The cytoplasmic sap did not incorporate radioactive phosphate into its RNA to a significant extent. Even though the microsomal RNA was decreased nearly to zero by the 2nd day in the radicle, a large amount of the residual microsomal RNA was labeled with radioactive phosphate, suggesting a rapid turnover.

GROWTH & ADP-C_8^{14} UPTAKE BY EXCISED TISSUES: Root and mesocotyl sections incubated under suitable conditions increased in fresh weight 13 and 15%, respectively, in an 8-hour period (table III). The increased weight of the scutella (3%) was probably due to hydration. The root and mesocotyl tissues lost soluble nucleotides during the incubation period with no change in the soluble nucleotide content of the scutellum. None of the tissues had a net gain or loss in RNA.

All three of the tissues appeared to accumulate large amounts of ADP-C_8^{14} into the acid-soluble fractions. Root tips accumulated more ADP-C_8^{14} than did either the mesocotyl or scutellum tissues. However, the scutellum tissue accumulated three times more ADP-C_8^{14} than did the mesocotyl sections.

Even though there was no net gain in RNA in any of the tissues, the root tips and scutella incorporated a large amount of ADP-C_8^{14} into their RNA-s. The ratios of ADP-C_8^{14} incorporated to accumulated were 1:7, 1:12, and 1:6 for root tips, mesocotyl sections, and scutella, respectively. Although the mesocotyl tissue accumulated less (67-75%) ADP-C_8^{14} than either scutellum or radicle tissues (table III), it was even less efficient in incorporating ADP-C_8^{14} into RNA.

SEPARATION OF ORGANIC PHOSPHATES & PHOSPHORIC ACID BY COLUMN CHROMATOGRAPHY: An

elution-chromatogram of the ultraviolet absorbing and P^{32} labeled components from 3-day-old radicle tissue which had been grown in a radioactive phosphate medium is given in figure 4. Similar elution-chromatograms were obtained from 3-day-old scutellum tissue; however, due to the likeness to their radicle elution-chromatograms only the latter will be presented. The identification of the nucleotides was established by the order and position in which they were eluted from the column as previously described (3). All the commonly occurring nucleotides previously identified were labeled with P^{32} by the 3rd day.

In general, the radioactive peaks were much sharper than the ultraviolet peaks (O.D., 260 mμ) which suggests that other ultraviolet absorbing compounds, not nucleotides, are adsorbed to the Dowex and are eluted from it contaminating the nucleotide peaks and thus interfering with quantitative nucleotide measurements. Generally, UDP, ATP, and UTP contained most of the nucleotide P^{32} label. There were two radioactive peaks (P_1 & P_2) which did not correspond to any of the ultraviolet peaks. P_1 and P_2 contained 10 and 42 % of the total radioactivity added to the column, respectively. The composited eluates of P_1 and P_2 were each lypholyzed to dryness and co-chromatographed on paper using two different solvents (Methods). Each of the unknown samples (P_1 & P_2) contained only one component. The Rf values of P_2 in both solvents used were the same as that of phosphoric acid. The identity of P_2 as phosphoric acid was further verified by adsorbing radioactive phosphate on a Dowex–1 × 8 column using ADP as a marker and eluting with the same system as used for column chromatography of the acid-soluble nucleotides (3). Radioactive phosphoric acid was eluted from the column at the same tube number as was P_2.

The identity of P_1 was not established; however, its migration in both solvents was similar to the hexose monophosphates. Due to the small amount of P_1 obtained from the column, a sugar moiety was not determined. The position of P_1 on the elution-chromatogram suggests that it may be a sugar phosphate.

It is interesting that very little of the radioactive phosphate was incorporated into the nucleoside monophosphates. No trace of radioactivity was found in Compound XI which agrees with previous work (3) that Compound XI is not a nucleotide.

Discussion

The evidence gained from these experiments indicates that there is a dynamic change in the particulates of radicles and scutella as corn seedlings grow. The RNA measurements, which may be regarded as an index to particulate mass, show that the microsomes sedimenting at 110,000 *g* rapidly decrease with seedling growth (fig 2). The small microsomal particulates appear to be either completely degraded or attached to larger or similar particulates so that some sediment with mitochondria as the plant material matures. If the microsomes are attached to other particulates so that they have a mass similar to mitochondria or if they are attached to the mitochondria, it is not known whether or not the cytological function of the microsomes is also changed. Although the cytoplasmic particulates undergo a dynamic change, it seems unlikely that the microsomes are completely degraded since protein is synthesized on the 4th day. The rapid decrease in microsomal RNA with growth is probably due to the decline in number of active cells as compared to mature cells.

Investigations by Lund et al (9) clearly show that there are changes in cytoplasmic particulates during growth and maturation of root cells which correlate with changes in the composition and physiological activity of these cells.

The mitochondrial RNA of the scutellum and radicle tissues may be correlated with the changes of the mitochondria. Lund et al (9) reported that immature mitochondria develop cristae and reach maturity as roots grow. Hanson et al (7) reported that the mitochondrial mass of corn scutellum increased with germination for 3 days, then began to decline. During germination of corn seed the respiratory activity of the scutellum (7) increased for 3 to 4 days with a decline in respiration thereafter. There observations on the development of mitochondria in

Table III

Changes in Fresh Weight, Soluble Nucleotides, & RNA, & ADP-C_8^{14} Uptake by Scutellum & Root Tip & Mesocotyl Sections in 8-Hour Period

Tissue	Tissue growth % (increase)	μg/gm Fresh weight				ADP–C_8^{14} Uptake[1] (cpm/gm Fresh wt)	
		Soluble nucleotide		RNA			
		Initial	Final	Initial	Final	Soluble nucleotides	RNA
Root tips (1 cm)	12.8	1,630	1,375	2,290	2,260	11,875	2,033
Mesocotyl section (1 cm)	15.2	1,530	1,235	813	835	3,370	317
Scutella	3.0	959	963	2,170	2,185	9,210	1,980

[1] The sum of radioactivity in the soluble nucleotide and RNA fractions is considered as the amount accumulated.

corn scutellum can be associated with the increase in mitochondrial RNA (fig 2).

As corn seed germinates there is a steady decline in scutellum RNA and a synthesis of radicle RNA (table II). The loss of scutellum RNA is greater than the increase in soluble nucleotides. A possible explanation for the mode of RNA utilization is that the RNA is degraded to nucleotides and used for growth of the embryonic plant or that a transportable RNA is moved to the growing tissue where it is used.

Summary

The change in amounts of nucleotides and RNA in corn seedlings during germination has been studied. Normally, as corn seeds germinate there is a production of nucleotides in the scutellum and in the radicle tissues. Both scutellum and radicle tissues incorporated fairly large amounts of radioactive phosphate into the acid-soluble nucleotides with the radicle accumulating and incorporating radioactive phosphate at a faster rate than the scutellum.

RNA of the scutellum was rapidly degraded apparently at the expense of the microsomes. Mitochondrial RNA was highest on the 2nd and 3rd days with rapid degradation occurring on the 4th day. The amount of radioactive phosphate incorporated into the scutellum RNA-s appears to be due to exchange reactions rather than de novo synthesis.

Generally, all of the RNA of the radicles was in the mitochondria except for 1-day-old radicles which had about half of the RNA content in the microsomes. All of the radioactive phosphate incorporated into the cytoplasmic RNA-s in the radicles was ultimately localized in the mitochondria. The cytoplasmic sap RNA incorporated no radioactive phosphate.

The protein content increased in all the cytoplasmic fractions in the radicle from 1 to 4 days whereas the protein content in the scutellum cytoplasmic fractions decreased with germination.

The nucleotide content of excised root tips and mesocotyls decreased in an 8-hour incubation period with ADP-C_8^{14}. The scutellum nucleotides did not change in the same period. The RNA contents of the root tips, mesocotyl sections, and scutella remained the same throughout the incubation period, however, the root tips and scutella incorporated a fairly large amount of ADP-C_8^{14} into their RNA-s. Mesocotyl sections did not incorporate ADP-C_8^{14} into its RNA as well as root tips or scutella.

The organic phosphates and phosphoric acid labeled with P^{32} from 3-day-old radicle tissue were separated by ion-exchange chromatography. The nucleoside di- and triphosphates contained most of the nucleotide radioactive phosphate. Two radioactive peaks were separated that did not correspond to any nucleotide peaks. The first radioactive peak was not identified by paper co-chromatography but it is believed to be a sugar phosphate. The second peak was identified as phosphoric acid using paper co-chromatography and ion-exchange chromatography.

Acknowledgment

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FOLIAR PENETRATION BY CHEMICALS[1,2]

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The unperforated cuticle, breaks in the cuticle, and openings in the epidermis, such as stomata and hydathodes, are possible sites of entry for foliar-applied chemicals. The cuticle is a barrier to penetration, but it is not impenetrable. Cuticular penetration has been amply demonstrated by application of chemicals to the stomata-free surface of hypostomatous leaves (3, 14, 19) and by applications in the dark when the stomata were closed (10, 16, 20). Yet the exact nature of cuticular entry is not known. Some suggested preferential pathways include epidermal cells above veins (18), hairs (7), anticlinal walls of epidermal cells (14), guard cell walls (21), and tears or punctures (13).

The problem of stomatal penetration has received much attention in the past, and stomatal entry by oils is well known (5). However, evidence for entry by aqueous solutions is conflicting. Skoss (15) and others (3, 8, 9) have reported that aqueous solutions rapidly enter open stomata. On the other hand, many workers have concluded that there is no entry via this route (2, 16, 19, 20). The intermediate position is that of van Overbeek (18) and others (4, 12, 17) who state that entry may occur if an efficient surfactant is employed. The consistently reported failure of surfactants to enhance uptake of inorganic nutrients (1, 11, 16) contradicts this hypothesis.

MATERIALS & METHODS

A primary concern in the selection of methods was the detection of the penetrant at the cellular level. The methods chosen to fulfill this criterion were: A. use of a fluorescent tracer, B. use of radioactive tracers in combination with the fluorochrome, and C. a precipitation technique. Treatments were of short duration in order that penetration might be studied without interference from other processes such as translocation. It was also felt essential that the degree of opening of the stomata at the time of treatment be known and controlled. For this purpose, environmental conditions were controlled to favor opening, or collections were made at the proper time of day for the open condition. When leaves with closed stomata were desired, collections were made at night or from plants held in darkness. Direct microscopic examination was the usual method of estimating the number of open stomata and the degree of opening. The solvent injection method was used for species with thick leaves.

In studies by the fluorescent dye method (6), 0.1 % (w/v) solutions of the fluorochrome sodium 3-hydroxy-5,8,10-pyrenetrisulfonate were applied as droplets or by immersion of the test leaves in the dye solution. Penetration of surfactant-free solution was usually compared with that of solutions containing the anionic surfactant Vatsol OT (sodium dioctylsulfosuccinate). At the end of the test period, nonabsorbed dye was removed by washing in running water, and the leaves were examined and photographed in ultraviolet light. A relative measure of dye penetration was obtained by measuring the fluorescence intensity of the treated leaves with the Photovolt model 501-M photometer equipped with a filter to remove the ultraviolet wave lengths. The meter readings were corrected for the primary fluorescence of untreated leaves.

In studies using fluorescent dye and radioactive tracers in combination, the test solutions contained 0.1 % fluorochrome and 0.1 μc of radioactive isotope (specific activity c. one μc/mole) per 0.01 ml. A 0.01-ml droplet was placed on the lower surface of each leaf. After a 5 minute treatment period, the leaves were washed in running water. Photographs were taken showing fluorochrome distribution, and radioautograms were prepared to determine isotope location. In addition, the activity of each leaf was assayed with a thin window G-M tube and Tracerlab 1000 Scaler.

The third method used was the prussian blue precipitation method (14) for detecting the penetration and internal movement of iron. Zebrina leaves were immersed for 5, 15, 30, or 60 minutes in 10 % ferric sulfate with or without surfactant (Vatsol OT). The washed leaves were cut into centimeter sections and infiltrated under vacuum with 10 % potassium ferrocyanide. Freezing microtome sections were cut from tissue fixed overnight in dilute formalin. Leaves designated as controls were carried through the same procedure omitting the ferric sulfate treatment.

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[2] This work was supported in part by allotments under section 9b 3, Bankhead-Jones, Title 1, W-11 Project, Cal. Agr. Exp. Sta. Project 1583.

[3] The data reported here are taken from the Ph.D. thesis of C. Dean Dybing, presented to the Graduate Division, University of California, Davis, October 1958.

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Results

Cuticular Penetration: Cuticular penetration was studied by treatment of leaves having closed stomata. The species tested were *Zebrina pendula* Schnizl., apple (*Pyrus malus* L.), and bean (*Phaseolus vulgaris* L.). The leaves were held in the dark throughout the treatment period.

A very minute quantity of fluorescent dye was absorbed through both the upper and lower surfaces of Zebrina leaves in 15 minutes, but at least one hour was required for appreciable uptake. Vatsol apparently enhanced uptake slightly. The dye appeared first in the anticlinal epidermal walls. In the longer treatment periods (1–4 hr) it also appeared in the periclinal walls. Occasionally the guard cells and accessory cells were stained, and, since the stomata were closed, this result may indicate entry through the cuticle of these cells. Cuticular absorption of iron by Zebrina leaves was evidenced by the deposition of prussian blue in the anticlinal walls of the epidermis, in the veins, and in the leaf hairs. Penetration of iron was detected in treatment periods as short as 30 minutes.

The cuticle of apple and bean leaves was penetrated somewhat more readily by the fluorescent dye than the cuticle of Zebrina with appreciable fluorescent staining occurring in 30 minutes. In the case of apple leaves, the dye was located mainly in the leaf hairs, while bean leaves absorbed the dye first through the epidermal cells of the veins (fig 1). Brushing the bean leaves with a camel's-hair brush greatly enhanced dye absorption, and again the dye was mainly in the veins.

Stomatal Penetration by Chemicals Applied With Surfactants: The fluorescent dye rapidly entered the open stomata of Zebrina from solutions containing from 0.05 to 0.5 % Vatsol OT (figs 2 & 3). The absorption apparently was not due to an effect of light on cuticular permeability because leaves in the dark with open stomata (exposed to CO_2-free air to force opening) were penetrated while those in the light with closed stomata were not. Movement of the penetrant within the leaf tissues was also observed (figs 4 & 5). Immediately after treatment, the substomatal chambers were filled with fluorescent dye. The dye then moved via wall channels into the mesophyll and epidermis. Eventually it moved completely out of the substomatal chamber. Migration through the leaf from epidermis to epidermis was frequently observed. Iron in 0.5 % Vatsol OT penetrated Zebrina leaves through open stomata in 15 minutes and was detected in the substomatal chambers and in intercellular spaces in the mesophyll. Movement of iron in the walls was clearly indicated in epidermal and mesophyll tissues. Passage into protoplasts was also indicated.

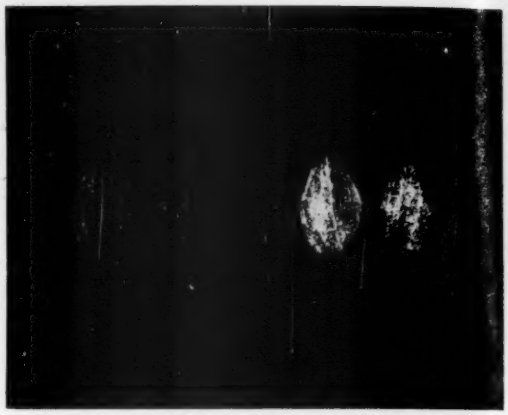


Fig. 2. Stomatal penetration of Zebrina leaves by fluorescent dye applied in 0.1 % Vatsol OT. *Left to right*: two leaves with stomata closed at time of treatment, untreated control leaf, and two leaves with stomata open at time of treatment. Treated 5 minutes.

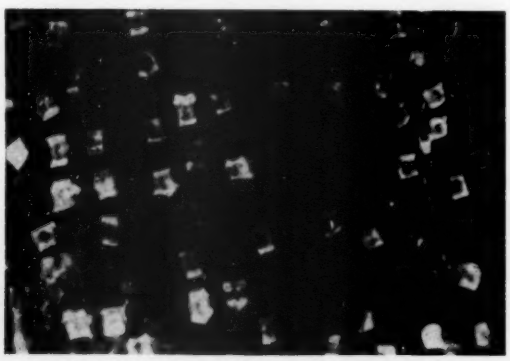


Fig. 3. Fluorochrome in the substomatal chambers of Zebrina leaf treated 10 minutes with dye solution containing 0.1 % Vatsol OT. About 30 ×.

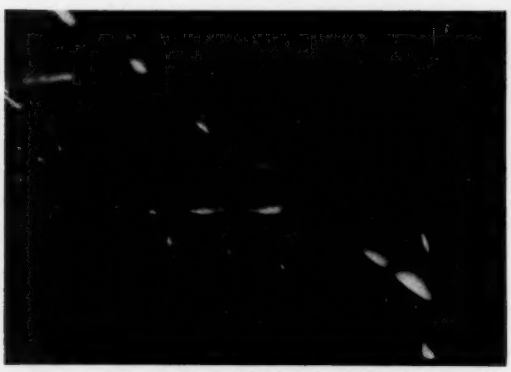


Fig. 1. Penetration of fluorescent dye into a bean leaf (lower surface) through the cuticle of hairs and epidermal cells over the veins. Treated 30 minutes. About 4×.

FIGS. 4 & 5. Movement of fluorochrome within Zebrina leaf. Figure 4 shows photomicrograph taken immediately after 5 minute treatment with surfactant-containing dye solution. Figure 5 shows the same area as seen 20 minutes later. The dye has moved from the substomatal chambers into the surrounding cell walls. About 20 ×.

To examine further the question of stomatal penetration by solutions containing surfactant, leaves of other species were subjected to the fluorescent dye test (table I). Every species tested showed stomatal penetration when treated for 5 minutes with dye solution containing 0.5 % surfactant. At surfactant concentrations lower than 0.1 % little or no stomatal entry could be detected with most species. However, the dye entered pear (*Pyrus communis* L.) and *Lactuca scariola* L. leaves (figs 6 & 7) at 0.05 % and *Chenopodium album* L. and apricot (*Prunus armeniaca* L.) leaves at 0.01 %.

In another test using apricot leaves, five surfactants were compared with Vatsol OT for efficiency in promoting stomatal penetration. Again the dye was absorbed in the 5 minute test period only when the stomata were open (table II). However, the amount of uptake varied with the surfactant used, Tween 20 being the least efficient of the six surfactants.

In the combination test radioactive tracers were

TABLE I

EFFECT OF VATSOL OT CONCENTRATION ON STOMATAL PENETRATION BY FLUORESCENT DYE IN A 5 MINUTE TREATMENT PERIOD

PLANT	STOMATAL APERTURE	PHOTOMETER READING (AV. OF 5 LEAVES) SURFACTANT CONC, (%)				
		0	0.01	0.05	0.1	0.5
Phaseolus vulgaris L. (trifoliate leaf)	Open	...	3	1	4	49
	Closed	...	3	7	3	6
Vicia faba L.	Open	2	0	0	80	443
	Closed	0	1	8	6	13
Prunus armeniaca L.	Open	8	12	146	171	203
	Closed	0	0	2	3	2
Pyrus communis L.	Open	0	4	110	218	267
	Closed	0	1	3	11	32
Citrus sinensis Osbeck	Open	0	0	9	22	70
	Closed	3	3	1	1	1
Sorghum halepense (L.) Pers.	Open	0	8	8	5	132
	Closed	0	0	0	0	0
Lactuca scariola L.	Open	1	0	13	124	136
	Closed	4	7	1	4	15
Vinca major L.	Open	4	2	5	19	118
	Closed	4	4	3	8	9
Chenopodium album L.	Open	32	24	25	36	77
	Closed	1	0	8	1	0
Convolvulus arvensis L.	Open	4	3	0	2	129
	Closed	0	0	4	3	2

FIGS. 6 & 7. Stomatal absorption of fluorochrome by leaves of *Lactuca scariola* L. The dye solution contained, from left to right in each figure, 0.5, 0.1, 0.05, 0.01, and 0 % Vatsol OT. The treatment time was 5 minutes. The leaves shown in figure 6 had open stomata at the time of treatment; those in figure 7 had closed stomata.

applied to the lower surface of Zebrina leaves for 5 minutes in 0.01 ml drops containing 0.1 % fluorochrome with or without 0.1 % Vatsol OT. By far the most absorption occurred in the open stomata-surfactant treatment (table III), and the dye and isotope distributions coincided in every replication of this series (figs 8 & 9). Photomicrographs revealed that the dye was located in substomatal chambers and in epidermal cells and anticlinal walls surrounding the chambers (fig 10). Leaves with closed stomata and those treated with surfactant-free solution absorbed no fluorescent dye and, with the exception of P^{32}, little or no radioactive tracer. Leaves with closed stomata were penetrated by P^{32} but uptake by those with open stomata from surfactant-containing solution was markedly greater.

STOMATAL PENETRATION BY CHEMICALS APPLIED WITHOUT SURFACTANT: In general, little penetration was observed in the foregoing tests unless a surfactant was used. To examine further the question of stomatal entry by chemicals applied in surfactant-free solutions, leaves of Zebrina, pear, and apricot were immersed for 30 minutes in surfactant-free solutions.

TABLE III

PENETRATION OF RADIOACTIVE CHEMICALS INTO ZEBRINA LEAVES IN A 5 MINUTE TREATMENT PERIOD

CHEMICAL	NET COUNTS/MIN (AV. OF 5 REPS.)			
	0.1 % VATSOL		NO VATSOL	
	STOMATA OPEN	STOMATA CLOSED	STOMATA OPEN	STOMATA CLOSED
C^{14}-labeled				
Urea	234	2	6	4
3-amino-1,2,-4-triazole	203	38	42	8
2,4,5-T*	115	8	8	12
Benzoate*	89	3	4	3
2,4-D*	127	13	18	6
2,4-D**	117	8	4	3
$H_3P^{32}O_4$	787	321	155	175***

* Triethanolamine salt

** Sodium salt

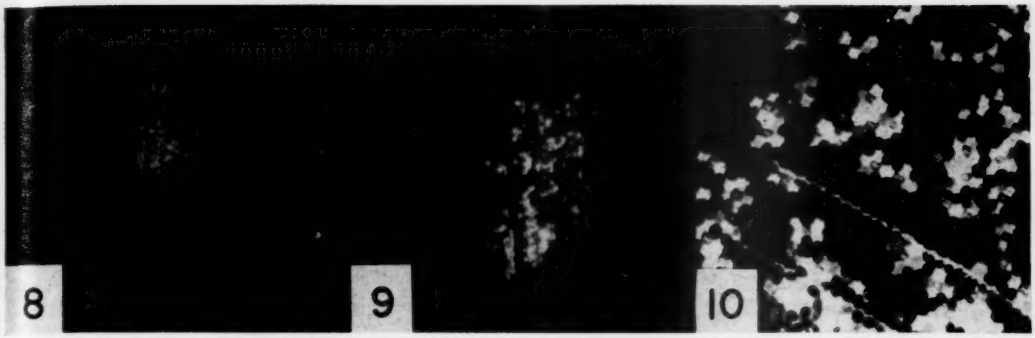
*** Four replications

TABLE II

EFFICIENCY OF DIFFERENT SURFACTANTS IN PROMOTING STOMATAL PENETRATION OF FLUORESCENT DYE INTO APRICOT LEAVES*

SURFACTANT, 0.1 %	PHOTOMETER READING (AV. OF 5 REPS.)	
	STOMATA OPEN	STOMATA CLOSED
Vatsol OT (Na dioctylsulfosuccinate)	468	9
Tween 20 (Polyoxyethylene sorbitan monolaurate)	235	19
X-77 (Alkylarylpolyethylene glycols plus free fatty acids & isopropanol)	423	9
Brij 30 (Polyoxyethylene lauryl ether)	375	5
Nonic 218 (Polyethylene glycol tertdodecyl thioether)	305	6
Tergitol NPX (2-ethylhexylphenyl ether of polyethylene glycol)	317	6

* Treatment period 5 minutes



FIGS. 8, 9, & 10. Fluorescent dye penetration compared with that of C^{14}-labeled 2,4,5-T (triethanolamine salt) applied in the same solution. A 0.01 ml droplet containing 0.1 % Fluorochrome, 0.1 % Vatsol OT, and 0.1 μc labeled 2,4,5-T was applied for 5 minutes to the lower surface of a Zebrina leaf. Figure 8 shows the distribution of the penetrated dye (c. 2 ×) and figure 9 shows the distribution of the radioactive tracer (c. 2 ×). Figure 10 is a photomicrograph showing the dye location at the cellular level (c. 10 ×).

Very little fluorescent dye was absorbed without surfactant by Zebrina or pear leaves in 30 minutes regardless of the stomatal condition (table IV). However, stomatal absorption occurred in all tests with apricot leaves. Surface tension measurements showed that during 30 minutes of immersion, sufficient surface active substance(s) escaped from the ten apricot leaves to lower the apparent surface tension of the treatment solution from 74.6 dynes per cm to 63.1 dynes per cm, a tension similar to very low concentrations of Vatsol. The substance(s) could not be completely removed by washing the leaves for 3 minutes before application of the dye.

When Zebrina leaves were treated for 30 or 60 minutes with surfactant-free iron sulfate solution, the iron was most frequently located in the anticlinal and periclinal walls of the epidermal cells proper. In some cases the guard cells and accessory cells were heavily stained also, but no precipitate formed in the substomatal chambers and the mesophyll beneath the stomata.

DISCUSSION & CONCLUSIONS

In these studies cuticular penetration by the fluorochrome was slow with one to four hours being required for appreciable entry through either surface of Zebrina leaves. The surfactant Vatsol OT somewhat enhanced dye entry, possibly because of improved contact. Entry apparently took place through the cuticle over the anticlinal epidermal walls and possibly through the guard cell and accessory cell walls. Bean leaves readily absorbed dye through the epidermal cells (including hairs) above the veins, and the hairs of apple leaves were penetrated quite rapidly. The results obtained with radioactive tracers indicated that some chemicals, especially phosphate, penetrate the cuticle more rapidly than the fluorochrome. However, the demonstration that a relatively large polar molecule such as the fluorescent dye used here can penetrate the plant cuticle is important.

Stomatal penetration by chemicals applied in surfactant-containing solutions has been demonstrated in these studies with all of the species and all of the chemicals tested. The positive results obtained with every tracer tested would appear to indicate entry of the solution in bulk through the open pore rather than entry through the cuticle and walls of the guard cells.

All of the surfactants tested greatly enhanced stomatal penetration, but at any given concentration they varied in their ability to promote entry. With Vatsol OT, the amount of fluorochrome absorbed in 5 minutes varied widely with the species tested, and increasing concentration from 0.01 to 0.5 % resulted in increased uptake. Leaves of all test species were penetrated at the 0.5 % concentration, and those of several species, including Zebrina, were penetrated at 0.05 %. The stomata of bean leaves, a common test plant in penetration studies, were penetrated only at the 0.5 % concentration. Even then entry was low in relation to other species, probably because of the small size of the stomata.

The surfactant Vatsol OT was shown to enhance penetration by promoting stomatal absorption of the inorganic nutrients P^{32} and iron by Zebrina leaves. This result conflicts with reports of other workers

TABLE IV

ABSORPTION OF SURFACTANT-FREE FLUORESCENT DYE SOLUTION IN A 30 MINUTE TREATMENT PERIOD

PLANT	STOMATAL APERTURE	PHOTOMETER READING (AV. OF 10 REPS.)
Zebrina pendula Schnizl.	Closed	6
	Open	7
Pyrus communis L.	Closed	21
	Open	34
Prunus armeniaca L.	Closed	11
	Open	101

(1, 11, 16). The difference may be due to differences in test species and methods employed.

In contrast to the results obtained with surfactants, stomatal penetration tests conducted without surfactant failed to yield conclusive results. Entry of fluorescent dye could not be clearly demonstrated in 30 minute tests with three species (in the case of apricot, a surface-tension reducing material was present on the leaf itself). On the other hand, entry via the stomata cannot be completely negated on the basis of these tests. Perhaps experiments of longer duration would have presented more definite evidence on this question.

These results support the hypothesis that stomatal penetration occurs mainly when an efficient surfactant is used. It appears that the question, "Can an aqueous solution penetrate leaves through the open stomata?" cannot be answered simply yes or no. Such entry varies with the surfactant used, the concentration of surfactant, and the species in question. It must be concluded that promotion of stomatal penetration is a very important surfactant function.

Summary

The problem of foliar penetration has been investigated by use of fluorescent and radioactive tracers and by a precipitation method. The penetrants tested included both herbicides and nutrients.

Cuticular penetration occurred, but, with the exception of P^{32} phosphate, entry via this route was relatively slow. Stomatal penetration by aqueous solutions occurred rapidly if an efficient surfactant was used at the proper concentration. Surfactants varied in their ability to promote stomatal entry, and the concentration of surfactant necessary for stomatal penetration varied with the species being tested. The leaves of *Zebrina pendula* Schnizl., *Pyrus communis* L., *Prunus armeniaca* L., and *Lactuca scariola* L. were readily penetrated via the stomatal route. *Phascolus vulgaris* L. leaves, however, required a greater concentration of surfactant for stomatal entry, and cuticular penetration through areas over the veins took place quite rapidly. Stomatal penetration by surfactant-free solutions has not been clearly demonstrated in these tests.

Acknowledgments

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ACCUMULATION & TRANSFORMATION OF SUGARS IN STALKS OF SUGAR CANE. ORIGIN OF GLUCOSE & FRUCTOSE IN THE INNER SPACE[1]

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Previous work (1–4) has shown that movement of sugars into tissue slices from sugar cane stalks occurs by two processes. The first, movement into the outer space, is a diffusion process, while the second consists of metabolically-dependent accumulation into the inner space of the tissue. It was postulated (4) that the outer space consists of cell walls and cytoplasm and the inner space is the vacuole. It was shown that tissues immersed in low concentrations of C^{14}-labelled glucose, fructose, or sucrose accumulated C^{14}-labelled sucrose into the inner space, with only minor amounts of the label in glucose and fructose. Similar results were observed in storage tissues of intact plants when leaves were exposed to $C^{14}O_2$ in direct sunlight.

The immature internodes of sugar cane stalks, to which the photosynthetically active leaves are attached, contain considerable quantities of reducing sugars. For example, in internodes 6 and 7 (combined sample) of variety H37/1933, the reducing sugar content was 0.11 M and the sucrose content 0.05 M (Burr, private commun.). In commercial varieties the sucrose concentration of mature internodes is about 0.5 M, while the reducing sugar content is very low—about 0.015 M. A possible explanation for the presence of glucose and fructose in relatively high concentration in immature tissue is that these sugars are stored when the concentration of the photosynthate moving from the leaves exceeds the capacity of the tissue to accumulate sugar as sucrose. Among alternative possibilities, the rate of inversion of sucrose in the inner space would be a likely controlling factor.

It will be shown that sucrose is virtually the sole product of accumulation into the inner space and that reducing sugars arise by subsequent inversion. Additional observations on pathways in the accumulation process also are presented.

MATERIALS & METHODS

The sugar cane variety Pindar was used throughout. Tissue discs were cut from young internodes about five centimeters long. These would correspond to the fourth or fifth internode of variety H37/1933 used previously (4) and are less mature than the tissues used by Bieleski (1). The tissue was chosen because of its high reducing sugar to sucrose ratio.

Methods of cutting and handling the tissues and isolating, identifying, and assaying radioactive sugars were the same as before (4). The dimensions of the tissue discs were 6.0 mm diameter, 0.50 mm thickness. Quantitative determinations of sucrose and fructose were made by the method of Roe, Epstein, and Goldstein (6), and glucose by the method of Wilson (7). The sugars were separated chromatographically prior to analysis.

RESULTS

EFFECT OF SUGAR CONCENTRATION ON AMOUNTS OF SUCROSE & REDUCING SUGARS ACCUMULATED INTO INNER SPACE: C^{14}-Labelled sucrose or glucose was supplied to storage tissue from immature internodes and the distribution of label in the accumulated sugars determined after removal of the outer space sugar (tables I & II). It was found that the concentration of sugar in the external medium over a 700-fold

TABLE I

EFFECT OF SUCROSE CONCENTRATION ON DISTRIBUTION OF C^{14} IN SUGARS OF INNER SPACE OF TISSUE DISCS INCUBATED IN C^{14}-LABELLED SUCROSE

EXPT.	SUCROSE CONC, μmole/ml	DISTRIBUTION OF LABEL, %		
		SUCROSE	GLUCOSE	FRUCTOSE
1	292	42	7.5	7
	56	44	6	5
	6.8	44	4	5
	3.9	46	3	3
	1.0	39	4	3
2	535	64	7.5	8
	292	43	7.5	7.5
	29	50	4	4
	14.7	48	5	4.5
	2.9	57	3.5	2.5
	1.6	60	4	4
	0.84	58	3.5	3.0

Tissue discs were cut from immature internodes of v. Pindar, washed, and immersed in C^{14}-labelled sucrose solutions for 4 hours at 30° C. The outer space sugars were removed by washing in running tap water for 1 hour, and the inner space sugars extracted in 3 volumes 96 % ethanol.

[1] Received manuscript August 29, 1960.

TABLE II

EFFECT OF SUGAR CONCENTRATION ON DISTRIBUTION OF C^{14} IN SUGARS OF INNER SPACE OF TISSUE DISCS INCUBATED IN C^{14}-LABELLED SUCROSE OR GLUCOSE

SUGAR IN MEDIUM	CONC, μmole/ml	DISTRIBUTION OF LABEL, %			
		ORIGIN*	SUCROSE	GLUCOSE	FRUCTOSE
Sucrose	292	55	34	3.7	2.6
	29.2	37	48	3.2	2.5
	2.92	35	62	2.0	1.8
	1.59	41	58	2.2	1.5
Glucose	555	14	66	14	2.3
	60.6	8	80	7.4	1.8
	5.55	11	74	2.3	1.2
	2.66	23	69	2.5	1.2

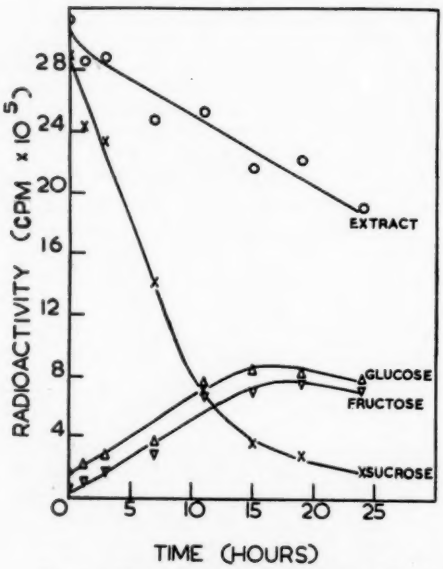
* Radioactivity in spot which remained at origin of paper chromatograms developed in ethyl acetate, pyridine and water (8:2:1).

Tissue discs were cut from immature internodes of v. Pindar, washed, and immersed in solutions of C^{14}-labelled sugars for 3 hours at 30° C. The outer space sugars were removed by washing in running tap water for 1 hour and the inner space sugars extracted in 3 volumes 96 % ethanol.

range did not have a very marked influence on the relative amounts of sucrose, glucose, and fructose in the inner space. At the highest concentrations of sucrose and glucose supplied (584 & 555 μmole/ml, resp.), 80 % or more of the label in the sugars was in sucrose. Consequently, the hypothesis that reducing sugars are stored when the concentration of sugar in the outer space exceeds some critical value is untenable.

FATE OF SUCROSE ACCUMULATED INTO INNER SPACE: Tissue discs were cut, washed, and immersed in glucose-C^{14} for 4 hours to accumulate labelled sugars in the inner space. The discs were then removed from the medium containing glucose-C^{14} and washed in unlabelled glucose for a 1-hour period to remove the labelled sugar from the outer space. A sample of discs was taken for analysis and the remainder placed in unlabelled glucose to accumulate sugar for a further 24 hours, samples being taken at intervals during this period.

The time course of movement of label in the sugars (fig 1) showed that loss of label from sucrose was accompanied by an increase in the labelling of glucose and fructose. Initially, 93 % of the total activity was in sucrose and 7 % in glucose and fructose. After 19 hours, 52 % of the initial activity had moved into glucose and fructose and less than 9 % remained in sucrose. Isolation of the sucrose from the initial tissue sample, followed by hydrolysis and radioactivity determinations, showed that the glucose and fructose moieties were equally labelled. The equal distribution of the label is reflected in the parallel increase of radioactivity in glucose and fructose from the



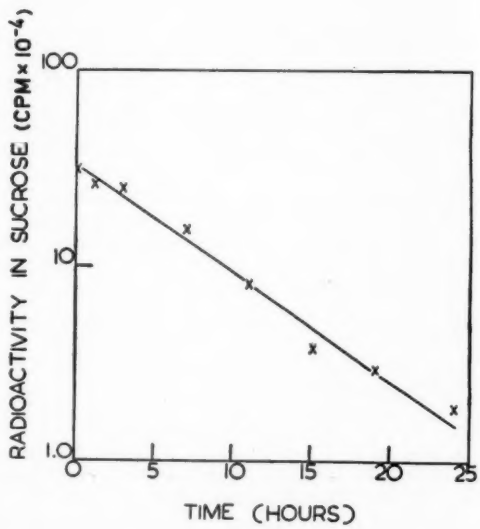


FIG. 1. Movement of C^{14} in sugars of the inner space. Storage tissue discs were placed in a solution of glucose-C^{14} for 4 hours, then washed in running tap water for 5 minutes and unlabelled glucose solutions for 55 minutes to remove labelled sugars from the outer space. Subsequently, the discs were incubated 24 hours in a solution of unlabelled glucose. Glucose concentration 6 μmole/ml throughout; temperature 30° C. Tissues were extracted in 3 volumes 95 % ethanol.

FIG. 2. Movement of C^{14} from sucrose of the inner space under steady-state conditions. Experimental detail as described for figure 1.

inner space, which is to be expected if these sugars originate from hydrolysis of sucrose.

Quantitatively, it was found that the sucrose, glucose, and fructose concentrations in the discs did not change significantly over the 24-hour period, so that approximately steady-state conditions prevailed during the time course of the experiment. The concentrations were 2.8 μmoles sucrose and 10 μmoles each of glucose and fructose per 25 discs.

The movement of label from the sucrose pool in the inner space may be described by the equation

$$\frac{-dS^*}{dt} = \frac{(R_1+R_2)S^*}{S} \qquad \text{I}$$

where: S^* is the radioactivity in sucrose; S is the sucrose concentration in the inner space; R_1 is the rate of transfer of sucrose into the reducing sugar pool; R_2 is the rate of transfer of sucrose to the outer space.

Integration of equation I gives

$$\frac{\ln S_0^*}{S^*} = \frac{R_1+R_2}{S}.\ t \qquad \text{II}$$

For steady-state conditions a plot of log S^* against time should be linear with a negative slope. The results plotted in figure 2 show that this expectation is fulfilled.

The half-time for movement of label from the sucrose pool is given by

$$t_{½} = 0.693\ \frac{S}{R_1+R_2} \qquad \text{III}$$

The half-time is about six hours (fig 1) and the turnover time (1.44 $t_{½}$) about 8.6 hours.

At a steady state for the sequential steps

$$\text{precursor} \rightarrow \underset{}{\text{sucrose pool}} \xrightarrow[k']{} \underset{}{\text{glucose + fructose pool}} \xrightarrow[k'']{} \text{product}$$

the rate of entry of sugar into the glucose and fructose pool is equal to the rate of loss. Assuming that all of the sucrose is disseminated by this pathway (tonoplast impermeable to sucrose; $R_2=O$), R_1/S estimates the velocity constant K′ and may be calculated from equation III, the value being 0.116 hours^{-1}. The sucrose content of the inner space (S) was 2.8 μmoles/25 discs, consequently the inversion rate (R_1) was 0.325 μmoles/hour. The glucose and fructose contents were each 10 μmoles/25 discs, therefore k″ was 0.0325 hours^{-1}, and the turnover time for the glucose and fructose pool was 31 hours.

From figure 1 it may be estimated that a minimum of about two-thirds of the sucrose passed through the glucose and fructose pool. Therefore, the maximum turnover time was approximately 46 hours.

Pathways of Sugar Accumulation: Bieleski (1) showed that the kinetics of sugar uptake into tissue slices from sugar cane internodes corresponded to those for enzymes described by the Michaelis-Menten system. The rate of accumulation increased with concentration in the external medium up to a limiting concentration at about 0.2 % (w/v). This work was confirmed independently by the author. However, in earlier work the tissues used were from somewhat more mature internodes than those in the current experiments which were chosen for high reducing-sugar content. For this tissue, divergence from the

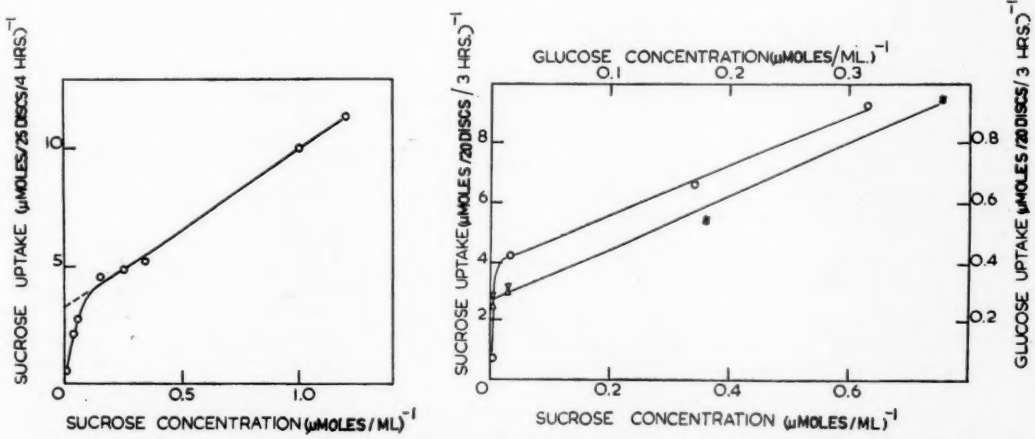


Fig. 3. Sucrose uptake in tissue discs from young internodes. Twenty-five discs per treatment were immersed in sucrose-C^{14} solutions (1.0 ml) for 4 hours at 30° C. Outer-space sugars were removed by washing in running tap water for 1 hour and the tissues extracted with 3 volumes 95 % ethanol. Sucrose uptake was measured by determining radioactivity in the extract.

Fig. 4. Comparison of uptake rates from glucose and sucrose solutions for tissue discs from young internodes. Experimental procedure described in table II. ○, sucrose uptake; △, glucose uptake; ▽, glucose uptake corrected for second process (see text for explanation).

Michaelis-Menten relationship was observed when high levels of sucrose were supplied in the external medium (fig 3). An estimate of the Michaelis constant from the slope of the linear portion of the curve at low sucrose concentrations gave a value of 2.2×10^{-3} M. Previous estimates were 2.5×10^{-3} M for the same variety (1), and 3.0×10^{-3} M (author, unpubl.).

In figure 4 the data for sugar uptake for the experiment described in table II are plotted and show divergence from the Michaelis-Menten relationship at high sucrose concentrations, but little divergence at high glucose concentrations even though glucose was supplied at double the maximum sucrose concentration. The initial sugar concentrations were 19 μmoles sucrose, 12 μmoles glucose and 6.4 μmoles fructose per 20 discs.

Where the tissues accumulated sugar from a medium containing 555 μmoles/ml glucose-C^{14}, the radioactivity in glucose in the inner space was six times that for fructose (table II). However, hydrolysis of the sucrose accumulated showed that 58 % of the label was in glucose and 42 % in fructose. Assuming the activity in fructose is a measure of the amount of reducing sugar derived from inversion of sucrose, 10.8 % of the labelled glucose entered the inner space by some alternative pathway. At lower glucose concentrations, entry by the alternative pathway was a negligible component of total glucose uptake. The results for glucose uptake in figure 4 are plotted both uncorrected and corrected for glucose movement by the second pathway. The Michaelis constant for sucrose uptake at low sucrose concentrations was 2.1×10^{-3} M and is comparable with values obtained from more rigorous data. For glucose uptake, the Michaelis constant was 7.0×10^{-3} M, compared with a value of 1.7×10^{-3} M obtained for more mature tissue (1). Thus the rate-limiting reaction for uptake at low sucrose concentrations appears to be at the same site over a considerable range of tissue maturity, while that for glucose may shift to some other step in a reaction sequence as the tissue ages. Because the rate of sucrose uptake increases sharply when the concentration approaches the saturation level for the low concentration reaction, a second pathway in parallel with the first is postulated.

Discussion

The hypothesis put forward previously to explain the results obtained on sugar uptake and accumulation (4) has been extended to include the results recorded herein (fig 5). Some of the more important evidence for this scheme follows.

I. If the rate-limiting reactions are postulated to be carriers taking sugar into the cytoplasm, there would need to be two carriers for sucrose, one being rate controller at low concentrations of sucrose and the second taking over as the concentration increased. One or more separate carriers for glucose and fructose would be required to account for the variable glucose to sucrose uptake ratio reported previously (4). As each sugar interferes with the uptake of the other sugars (1), competition at the carrier sites would have to occur. The rate of efflux of labelled sugars from the inner space is substantial and is not effected by the presence of unlabelled sugar in the medium (4). Consequently, it appears necessary to postulate a further set of carriers to mediate efflux independently of influx. The hypothesis that sugar uptake is limited by carriers at the boundary of the outer space and the cytoplasm has been discarded in favor of the much simpler hypothesis that sugars move freely into the tissue zone (presumed to be the cytoplasm) where sugar transformations occur. Hence the cytoplasm is part of the outer space where outer space is defined as the tissue volume which comes to rapid diffusion equilibrium with sugars in the external solution (the outer & inner space for this tissue may be quite different for solutes other than sugars).

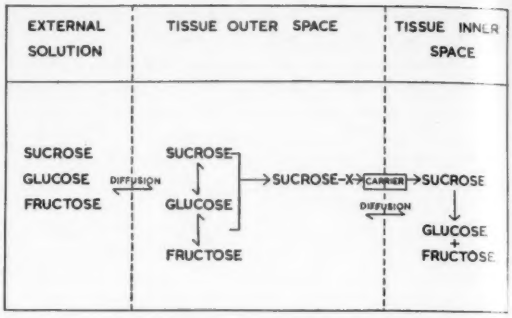


FIG. 5. Schematic representation of sugar interconversions and accumulation in sugar cane storage tissue.

II. Glucose is converted to sucrose prior to accumulation into the inner space (as shown by non-utilisation of the inner space fructose pool in sucrose synthesis, and the fact that the reducing sugar pool has its origin in the inversion of sucrose). As glucose can be taken up by the tissue and accumulated (as sucrose) at rates ten times higher than for equivalent concentrations of sucrose (4), it follows that sucrose itself is unlikely to be the substance transported. Hence, in figure 5, an unknown sucrose derivative or precursor is assumed to move across the barrier separating the outer and inner space. The energy necessary to drive the reaction in the direction of sucrose accumulation is assumed to be derived from breakage of the sucrose-X bond. The differences in rates of uptake of glucose and sucrose are readily explained if glucose is a better source of sucrose-X than is sucrose.

III. There are parallel pathways for the formation of sucrose-X from sucrose. One pathway predominates at low sucrose concentrations in the outer space and has a rate-limiting step with a Michaelis constant of about 2.5×10^{-3} M.

IV. In short-term experiments the efflux of labelled sugar from the inner space is not altered significantly by the presence of unlabelled sucrose in the bathing solution (4). This result is consistent with the hypothesis that efflux is a diffusion reaction as shown in figure 5.

It should be pointed out that uptake is not synonymous with accumulation of sugar in the inner space, as products other than sucrose, glucose, and fructose are formed. The rate-limiting reactions observed for sugar uptake are probably one or other of the enzyme-catalysed reactions concerned with phosphorylation and interconversion of the sugars.

An observation which may be of considerable importance in determining the mechanism by which certain plants store large quantities of carbohydrate as sucrose is that inversion of sucrose in the inner space of immature internodes occurs at a very fast rate (Ca. 10 %/hr). At 100° C and pH 4.6, the hydrolysis of sucrose proceeds at the rate of 5.3 % per hour (5). It is concluded, therefore, that inversion is a catalysed reaction. The inner space for sugars has been tentatively identified with the vacuolar volume. Among other possibilities, inversion could occur through the presence of invertase in the vacuole.

Maturation of sugar cane storage tissue is accompanied by an increase in the sucrose/reducing sugar ratio from something less than one to greater than 150/1. At the same time, the capacity of the tissue to take up sugar falls 6-fold for sucrose and about 15-fold for glucose when measured independently of efflux and on a fresh weight basis (4). For sucrose to accumulate in the inner space it seems highly probable that maturation of the storage tissue is accompanied by loss or masking of invertase activity.

Summary

I. Changes in the concentration of sugar in the external solution over a 700-fold range have little effect on the ratio of sucrose to glucose and fructose accumulated into the inner space of sugar cane storage tissue discs.

II. Tracer studies show that the origin of glucose and fructose in the inner space is from the inversion of the inner space sucrose.

III. In tissue from young internodes, the turnover time for the sucrose pool was about 8.6 hours, and 31 to 46 hours for the glucose-fructose pool.

IV. Evidence is presented for two parallel pathways of sucrose uptake in young tissue, one being predominant at low sucrose concentrations and the other at high concentrations.

V. The conclusion is reached that a sucrose precursor or derivative is transferred across the boundary separating the outer and inner space of the tissue, and sucrose is released into the inner space.

VI. The maturation of storage tissue is accompanied by a marked increase in the sucrose content and concomitant decrease in glucose and fructose content. It is suggested that the inversion of sucrose occupies a key position in storage of sucrose in the inner space, and that the rate of inversion must decline as the tissues mature.

Acknowledgments

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EFFECTS OF WAX COATINGS ON LEAF TEMPERATURES & FIELD SURVIVAL OF PINUS TAEDA SEEDLINGS [1]

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This paper reports two experiments. The first was a field test of the effectiveness of wax foliage coatings in improving survival of loblolly pine (*Pinus taeda* L.) seedlings planted on dry sites. The second was a laboratory study of a suspected upset in the heat balance of wax-coated leaves.

The bulk of experimental evidence, as well as horticultural practice, supports the use of wax coatings for stock to be stored or transported. There is little agreement, however, that coatings can consistently improve the drought-resistance of growing trees. Coatings have materially reduced transpiration in some studies (4, 6, 10), but not in all (1, 8). In the field their net effects on survival sometimes have been beneficial (2, 11) and sometimes useless (7, 9, 12).

FIELD TEST. One-year-old nursery seedlings were planted near Oxford, Miss., in February 1957. The site was an actively eroding sandy loam with a moderate stand of bluestems (Andropogon spp.). The study design consisted of four randomized blocks of five treatment plots each. Tree spacing was 5 by 5 feet; each plot originally had 36 seedlings, but by first treatment in June the average was 34. A 25 % water emulsion of Dow-wax was the transpiration retardant.

Treatments were: Sprayed once. Sprayed four times. Sprayed once and shaded with cedar boughs stuck in the ground. Not sprayed but shaded with cedar boughs. Not sprayed or shaded. The first spray, as well as the shading, was applied on June 26. Repeat sprayings were made on July 16 and August 8 and 15.

RESULTS (FIELD TEST). The wax coatings had a highly significant adverse effect on survival. Mortality was generally proportionate to the number of sprayings. Survival of trees sprayed four times averaged 57 ± 15.7 %, as compared to 74 ± 2.9 % for trees sprayed once and 96 ± 1.5 % for untreated controls (fig 1). The cedar boughs lost most of their leaves by midsummer and had no significant effect on survival. Height growth varied from 0.64 to 0.74 foot among treatments, but none of the differences were significant.

Survival of the repeat treatment fell significantly behind that for the single application after the second spraying, but differences between the single applications and the control did not reach significance until September 8, after 40 days of drought. During this drought, soil moisture averaged 3.1 % by weight, 1.1 % below the 15-atmosphere tension value—an indication that soils were near the wilting point most of the time.

The generally good survival, despite the wax treatment and drought, may be accounted for by rooting depth. Roots of most seedlings extended below 18 inches in the loose sandy soil.

Waxed seedlings in shade had somewhat better survival than unshaded waxed seedlings. While the difference was not statistically significant, it suggested that the wax might have upset the heat balance between the seedlings and their environment under strong insolation.

LABORATORY STUDY. Ten 1-year-old nursery seedlings of loblolly pine were grown for 9 months in pots of silt loam soil. Then five were allowed to deplete soil moisture to near the wilting point and five were kept watered.

Temperatures were measured with butt-welded, 40-gauge (0.00313-inch diam) copper-constantan ther-

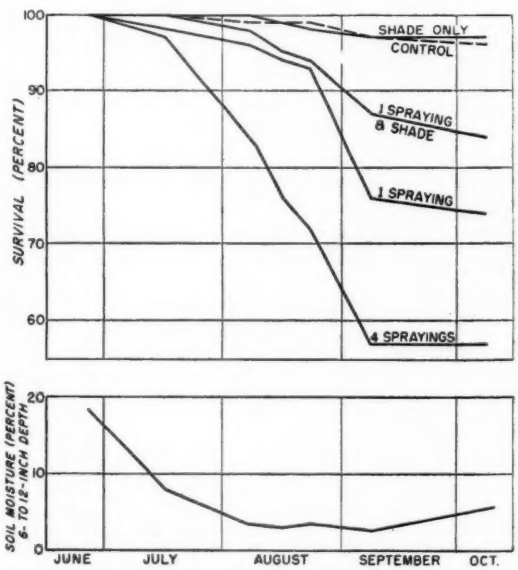

FIG. 1. Field survival and soil moisture content.

[1] Received September 6, 1960.

mocouples polished and threaded into two needles on each seedling. At least 1 cm of thermocouple was embedded in the needle on either side of the junction. The needles were individually held in a jig 8 inches from a 250 w incandescent lamp and the time required for each needle to reach constant temperature was recorded. The lamp was turned off and wax applied after the leaves had cooled back to equilibrium with the air. Because evaporation of the wax emulsion initially cooled the leaves 10 to 15° below air temperature, they were allowed to warm back to equilibrium before the lamp was turned on again.

The soil in each pot was sampled and needles of each seedling were clipped for determinations of moisture content.

Radiation received at the needle, as measured with a General Electric vacuum thermocouple radiation meter, was 1.1 gm cal./cm²/min. Laboratory air temperatures were between 78 and 80° F. Relative humidity varied from 24 to 38 %.

Results (Laboratory Study). Under the lamp, uncoated leaves reached equilibrium with the laboratory environment within 4 minutes, while waxed leaves continued to heat slowly after 7 minutes. Equilibrium temperatures of waxed leaves averaged 4.4° F higher than those for normal leaves; the difference was highly significant.

There were no significant differences between seedlings from dry and wet soils. Moisture content of the dry soils averaged 9.9 % by weight (0.3 % above the 15-atmosphere value), while the wet soils averaged 37.1 %. Moisture content of needles from seedlings on dry soils averaged 116 % of oven-dry weight, as compared with 192 % for needles from watered seedlings. At full turgor, needles averaged 232 % moisture.

Figure 2 shows the average increase in leaf temperature for the four treatments. The relationships between leaf temperature and time for all four treatments were fitted to a function of the form

$$Y = b_0 + \frac{b_1}{X_1} + b_2 X^2$$

where Y is the difference between leaf and air temperature and X is the number of minutes after heat is applied. Correlation coefficients for the $\frac{1}{X}$ variable were highly significant for all treatments, and ranged from 0.934 to 0.993. Coefficients for the x^2 variables were highly significant for both wax treatments, significant for the normal dry leaves, and not significant for the normal wet leaves.

Differentiating the time-temperature equations shows that heat absorption decreases from an infinite value to a minimum point for the normal curves in about 5 minutes (table I). Absorption decreases to a point of inflection for the waxed leaves at 7 to 8 minutes.

As figure 2 indicates, normal leaves from seedlings on dry soil behaved similarly to those from seedlings

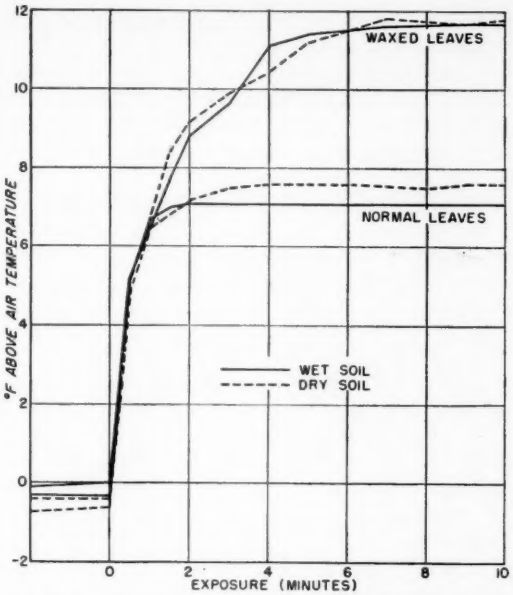

Fig. 2. Excess of loblolly pine needle temperatures above air temperature at radiation intensity of 1.1 g cal/cm²/min. Treatments represented by ten needles. At equilibrium, temperatures of replicate leaves did not vary more than 2.4° F from the mean of any of the treatments.

on wet soil—analysis of regression coefficients indicated no significant differences between the normal-leaf curves. Yet as the seedlings from wet soils were presumably transpiring much more rapidly than those from dry soil, the cooling effect of transpiration appears to have been minor—certainly not enough to explain the highly significant difference between waxed and unwaxed leaves. In other studies (3, 5, 13) the effect of transpiration on leaf temperatures was so small as to be overshadowed by the intensity of radiation and the angle of incidence, and by the emissivity of the leaves.

Since the mass of the leaves was not appreciably increased by the wax, their heat capacity was not altered. It seems possible that the coatings served as heat traps which permitted the passing of visible radiation but prevented much of the heat loss by

Table I

Instantaneous Rates of Change of Leaf Temperature With Time for Seedlings Growing in Wet Soils

Treatment	°F Time lapse (min)				
	1	2	3	4	5
Normal leaves	1.121	0.262	0.096	0.031	0.004
Waxed leaves	3.501	0.894	0.410	0.260	0.193

radiation in the infrared. Even a small rise in leaf temperature is accompanied by a large increase in the vapor-pressure gradient between leaf and atmosphere. Thus, any reduction in transpiration resulting from clogging of the stomata or interference with cuticular transpiration might be overbalanced by the greatly increased potential for moisture loss.

If possible toxic effects of the wax are discounted, disturbance of the heat balance between the trees and their environment may explain the heavy mortality of waxed seedlings in the Mississippi field study.

Summary

Survival of loblolly pine seedlings planted on a sandy soil in Mississippi was diminished when a wax transpiration retardant was applied once in late June, and was greatly reduced when treatments were repeated several times during the summer. Losses for both single and repeat treatments increased as the soil approached the wilting point. Height growth was unaffected.

Under radiant heat in the laboratory the temperature of waxed leaves rose an average of 4.4° F above that of normal leaves. Rates of heat absorption of the waxed leaves were considerably greater than for the normal leaves. The relationship of the increase in temperature with time under constant radiation for both waxed and normal leaves was found to be best expressed by the equation $Y = b_0 + \frac{b_1}{X_1} + b_2X^2$. The amount of water available for transpiration had no apparent effect on leaf temperature.

Mortality of treated seedlings is attributed to an unfavorable upset of the heat balance between leaves and atmosphere under high insolation.

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KINETICS OF RUBIDIUM ABSORPTION & TRANSLOCATION BY BARLEY[1, 2]

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Introduction

The transport of ions from a bathing solution to the top of a plant has been described as a passive (7, 8, 18, 19, 22, 23, 28) and active (2, 3, 17, 26, 27) process. Brouwer (3) states that at least 70 to 85 % of the ions appearing in the tops of plants go through an active process, i.e., "owing to a mechanism controlled or started by metabolism lands into the transpiration stream". On the other hand, Epstein (7) concludes that "the cells of the roots, as well as the shoots, derive their ions from the solution in the 'outer' space of the root tissue". He defined outer space as the "space to which inorganic ions have free and reversible access by diffusion".

The purpose of this study was to investigate these suggested concepts of ion passage from outside solutions to the top of the plant, and to formalize the observations with equations consistent with available data. The technique is kinetic analysis of ion uptake from solutions of radioactive rubidium ranging in concentration from 10^{-5} to 10^{-2} molar, in the presence and absence of inhibitors.

Kinetic Analysis

The method consists of analyzing ion uptake data under steady state conditions. Details are discussed elsewhere (10, 11, 12, 13). It is based on the assumption of a carrier concept for metabolic ion uptake as described by Epstein and Hagen (9) in equations A and B

$$R + M_{(outside)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MR \qquad A$$

$$MR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R' + M_{(inside)} \qquad B$$

where R represents a metabolically produced carrier, M, the ion, MR, the unstable carrier-ion complex, and k the rate constant for each reaction. The outside and inside referred to in Epstein and Hagen's equations A and B assumes carrier complex movement through a membrane but this is not a necessary requirement of the kinetics.

If k_2 is the rate limiting step in the forward direction and reaction B is essentially irreversible, the absorption of the ion is proportional to MR. The concentration of MR is determined by the steady state conditions. An equation derived, analogous to that used for enzyme action by Eadie (5) and Hofstee (15) in case III, relates ion absorption to ion concentration as shown in equation C,

$$v = -K_m \frac{v}{M} + V_{max} \qquad C$$

where v is the amount of ion absorbed, V_{max} is the amount absorbed when all the carrier is saturated with the ion under study, i.e. MR = R, and $K_m = \frac{k_{-1} + k_2}{k_1}$ and is the apparent dissociation constant of MR.

If v is plotted against $\frac{v}{[M]}$ a straight line should result for each site of uptake provided [M] remains essentially constant during the steady state uptake. The slope of this straight line, $-K_m$, is the Michaeles constant (24).

Methods

Seeds of Atlas 46 barley were aerated in demineralized water at 24° C for 24 hours in the dark. They were rinsed in demineralized water, placed in containers made by boring a 40 mm hole vertically through a no. 9 stopper and fixing a stainless steel screen across the bottom. Thirty-six of these containers were placed in holes in a masonite or aluminum sheet and positioned over a pyrex tray containing a nutrient solution with the following concentration in ppm: Ca–62, Mg–8, N–63, P–10, K–100. The seeds were placed on the stainless steel screen immediately above the nutrient solution. Cheesecloth was placed over the containers and in contact with the solution to prevent drying of the seeds. The cheesecloth was removed after 2 days and the nutrient solution renewed twice during the growth period. Plants were grown for a period of 6 days in a light chamber at 30° C and under a light intensity of approximately 1,800 ft-c. All the experiments were done in this same light chamber.

Solutions of RbCl were prepared and tagged with Rb^{86}. The pH was adjusted to 6.0 with either 0.1 N

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NaOH or 0.1 N HCl. The solutions were allowed to reach thermal equilibrium in the light chamber in which the experiments were performed.

At the beginning of the absorption studies each container of intact plants was removed from the growth trays and the roots rinsed in demineralized water. The container was placed in a fitted hole in a masonite cover that just covered the top of a 1 liter tall-form beaker filled with the Rb*Cl solution. To reduce evaporation, the junction between the masonite cover and the beaker was completely sealed with masking tape except for an aeration tube placed through a small hole in each cover. The entire assembly was then weighed with a precision of ± 10 mg with an analytical balance. The assemblies were set on a table in a random design and connected to a compressed air supply. A fan at the opposite end of the table circulated the air around the plants. At the end of the absorption period, the entire assembly was again weighed. The difference between these two weights is the water lost by transpiration and evaporation. The shoots were excised and weighed immediately. The roots were washed in four separate aliquots of demineralized water for a total time of 1 minute, excised, blotted on cheesecloth, and weighed. The containers then were placed back into the masonite covers, the entire assembly reweighed, placed in the original position on the table and connected to the air supply. The assembly was removed and weighed after a period of time equal to the absorption period. The loss of water during this period was considered to be evaporation. The amount evaporated subtracted from the amount transpired plus evaporated was considered the amount transpired.

After weighing, the plant roots and plant tops were placed in one-quarter ounce circular tin boxes. The roots were dried under an infrared lamp and the shoots ashed at 400° C. Aliquots of the radioactive solutions were pipetted into the same type tin boxes, dried, and used as a standard.

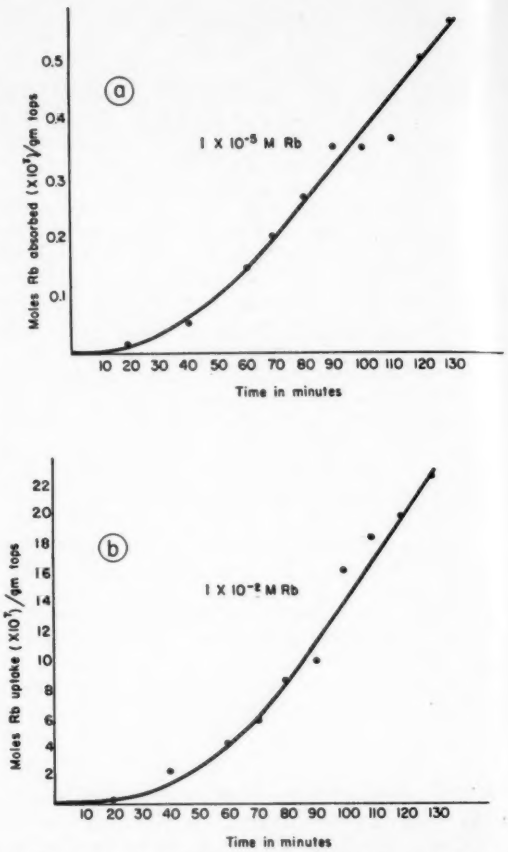


FIG. 1a & 1b. The rate of accumulation of Rb by shoots of 7-day-old barley plants from RbCl solutions of different concentrations. a) 10^{-5} M, b) 10^{-2} M.

RESULTS

Since steady state conditions are a necessary requisite for the kinetic analysis used, the attainment of linearity in a time curve of ions by the tops of plants was determined by time curves at all rubidium concentrations. Results of two separate experiments on rubidium accumulation from a 10^{-5} and 10^{-2} molar RbCl solution are shown in figure 1a and 1b. The results show a similarly shaped curve to that obtained by Helder (14) for shoots. Steady state was reached in about sixty minutes. Eighty minutes was therefore chosen as the time of absorption for the concentration curves.

Figures 2a and 2b show typical concentration curves where uptake of the accumulated ion is plotted on the ordinate and uptake divided by concentration on the abscissa as suggested by equation C. The concentration of rubidium chloride was varied from 10^{-5} to 10^{-2} molar. Figure 2a shows typical results for roots and figure 2b for the tops of the same plants. The results for roots show at least two sites of uptake consistent with previous observations by Fried and Noggle (10) and Bange (1). The results for shoots show a corresponding two sites indicating similarity in mechanism between the accumulation process in the roots and that in the shoots.

Although two sites were always evident, experimental conditions limited the number of treatments in any one experiment. Thus only seven different concentrations could be used. These are not enough to define both sites of uptake and only the site that predominates at lower concentrations were evaluated in these and subsequent experiments. However, 10^{-2} M RbCl was used in all experiments to represent the site that predominates at the higher Rb concentrations.

Many investigations (4, 9, 10) showed that potassium was competitive with rubidium in the active

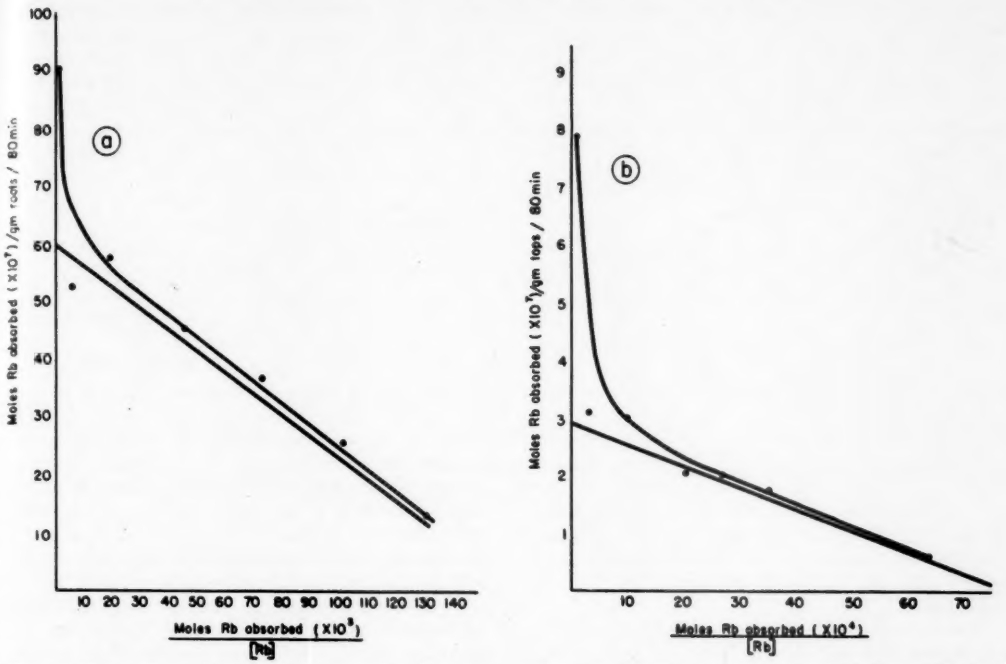


FIG. 2a & 2b. The effect of RbCl concentrations ranging from 10^{-5} to 10^{-2} M on the accumulation of Rb in 80 minutes by 7-day-old barley plants. a) roots, b) shoots.

accumulation process in roots. Figure 3 shows the results of a K competition experiment in accumulation of Rb by shoots again plotted according to equation C. When the site that dominates at lower Rb concentrations is delineated, the same V_{max} is observed both in the presence and absence of potassium. However, the slope is decreased in the presence of potassium. These observations are typical of a strict competition, again indicating a similarity in mechanism between shoots and roots.

Inhibitors—metabolic, like azide and methylene blue; uncouplers, like DNP; and competitors, like hydrogen ion—have also been shown to affect active ion accumulation in roots (3, 10, 13, 16, 20, 26). When measuring ion accumulation in shoots it is necessary to establish experimentally that the inhibitor does not affect transpiration since transpiration may affect ion accumulation. Sodium azide, 2×10^{-4} M, methylene blue 1×10^{-4} M, and DNP, 1×10^{-4} M, all at pH 6.0, and hydrogen ion at 1×10^{-4} M experimentally did not affect transpiration. Higher concentrations of these inhibitors did affect transpiration. Their effect on ion accumulation by the shoot is shown in table I. Both NaN_3 and DNP reduce rubidium uptake similarly at all concentrations, the DNP having the greater effect. Methylene blue also reduces uptake but only at the lower concentrations of rubidium, the effect disappearing at the high rubidium concentrations. Except at 1×10^{-5} M rubidium, hydrogen ion shows the type of inhibition of a competitor, i.e. the lower the rubidium concentration the greater the inhibition.

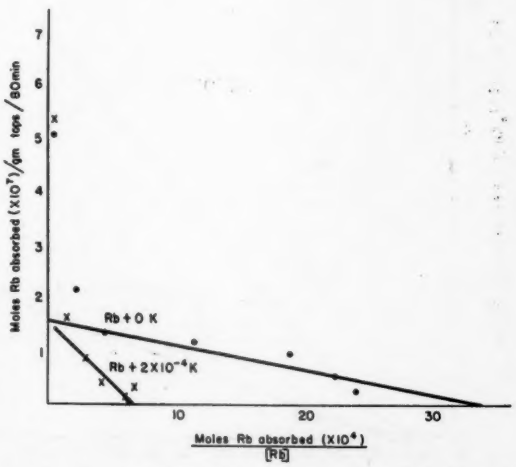


FIG. 3. The effect of 2×10^{-4} M K in solutions of RbCl ranging in concentration from 10^{-5} to 10^{-2} M on the shoot accumulation of Rb in 80 minutes by 7-day-old barley plants.

TABLE I

EFFECT OF 2×10^{-4} M NaN_3, 1×10^{-4} M DNP, 1×10^{-4} M MB ALL AT pH 6.0, & 1×10^{-4} M HYDROGEN ION ON Rb ACCUMULATION IN SHOOTS IN 80 MINUTES

MOLAR CONC OF RbCl	% OF CONTROL			
	NaN_3	DNP	MB	H+
1×10^{-5}	70	40	20	40
4×10^{-5}	50	30	30	20
7×10^{-5}	60	30	20	20
1×10^{-4}	80	40	40	50
3×10^{-4}	70	60	60	50
1×10^{-3}	60	40	90	50
1×10^{-2}	80	40	100	80

Different inhibitors affected Rb accumulation in shoots differently. Rubidium accumulation in roots was also measured in all the inhibitor experiments recorded in table I. Similar inhibitor effects on root and shoot accumulation invariably were found, again indicating similarity in mechanism for roots and shoots.

Time curves of the inhibition by 10^{-4} M DNP at 1×10^{-4} M Rb and 10^{-4} M MB at 5×10^{-5} M Rb are shown in figures 4a and 4b. The results of figures 4b were obtained on a different day and on a different set of plants than those in figure 4a. The time curves show that the inhibitor effects are accentuated with time. The inhibitor effect is most accurately determined by comparing the slopes of the time curves. These reveal that the slope differences are even greater than the uptake differences at 80 minutes.

Much evidence that ion accumulation from a bathing solution to shoots of plants is passive is based on the effect of transpiration on the appearance of the ion in the shoots (18, 19, 23, 29). The effect of transpiration rate was tested under the experimental conditions described under methods by varying transpiration without varying ion accumulation by roots. Two procedures were effective, turning out the lights during the experiment or increasing the osmotic concentration of the bathing solution by the addition of 2×10^{-1} M glycerine solution.

Figure 5 shows uptake by shoots in the light and in the dark at all concentrations between 10^{-5} and 10^{-2} M Rb. Turning out the lights had no significant effect on accumulation of Rb by roots. The results indicate that decreasing the transpiration by turning out the lights decreased Rb accumulation to approximately one-fifth of the light value but did not change the pattern of uptake. Thus, although transpiration may affect ion accumulation, the pattern of uptake is still similar to that of active accumulation in roots.

The results of similar transpiration experiments with glycerine are shown in table II and figures 6a and 6b. The glycerine had no significant effect on rubidium uptake by roots.

Time curves relating the effect of glycerine on transpiration to the effect of glycerine on ion accumulation are shown in figures 6a and 6b, where 6a

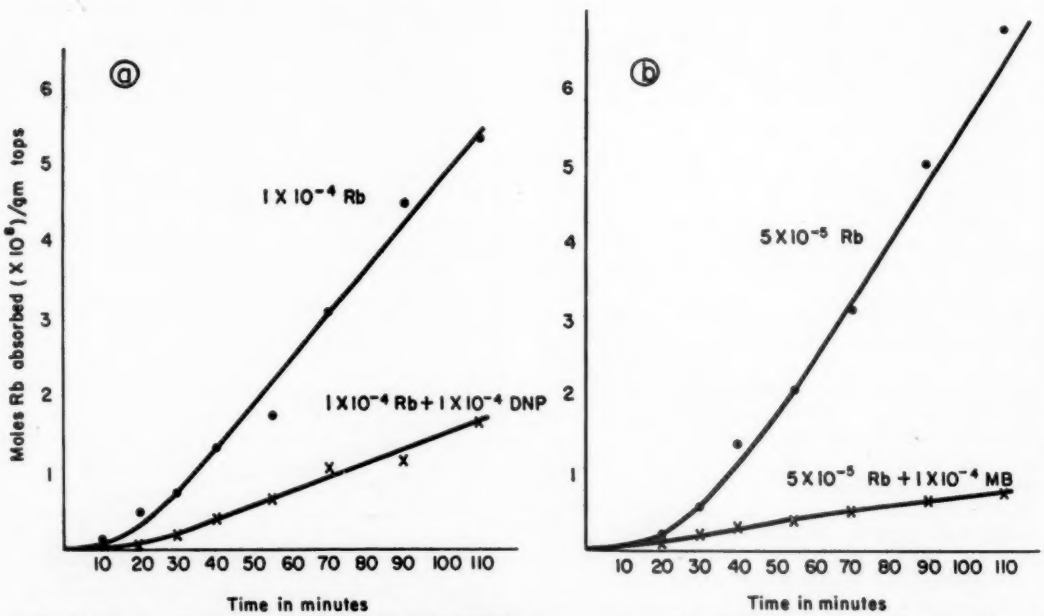


FIG. 4a & 4b. The effect of 2,4-dinitrophenol (DNP) and methylene blue (MB) on the rate of accumulation of Rb by 7-day-old barley plants. a) DNP, b) MB.

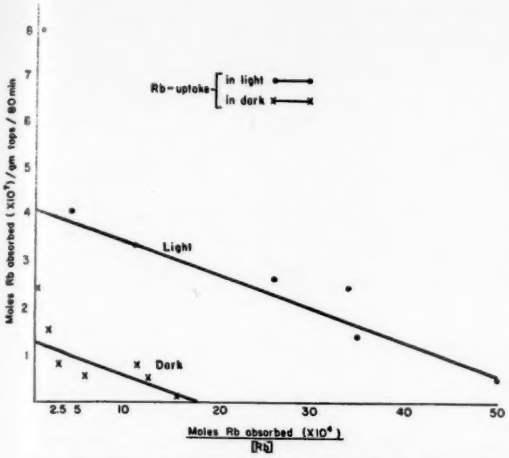


FIG. 5. The effect of darkness on the shoot accumulation by 7-day-old barley plants, of Rb from RbCl solutions ranging in concentration from 10^{-5} to 10^{-2} M.

shows the effect of glycerine on transpiration and 6b the effect of glycerine on ion accumulation by shoots. The valid comparison is between the slopes of the control and that of the treated plants. The results indicate an approximate equivalence of transpiration effect and ion uptake effect at a Rb concentration of 1×10^{-3} M.

The data in table II show results similar to the light and dark experiment, i.e., glycerine reduced uptake by shoots similarly at all concentrations and the concentration pattern therefore remained the same as for active accumulation in roots. The decrease in Rb uptake by shoots is slightly more than twofold. This is within experimental error of the decrease in transpiration which showed a ratio of 2.3 in the absence and presence of 2×10^{-1} M glycerine.

DISCUSSION

Ion accumulation from a bathing solution into and through the roots to the shoots of the plant may be described by at least four sets of equations, assuming a carrier mechanism for active ion accumulation in roots. Since active accumulation in the roots is

TABLE II

EFFECT OF 2×10^{-1} M GLYCERINE ON UPTAKE OF Rb BY SHOOTS IN 80 MINUTES

CONC OF RbCl moles/l	Rb ACCUMULATION % OF CONTROL
1×10^{-5}	45
4×10^{-5}	45
7×10^{-5}	55
1×10^{-4}	50
3×10^{-4}	45
1×10^{-3}	45
5×10^{-3}	55
1×10^{-2}	45

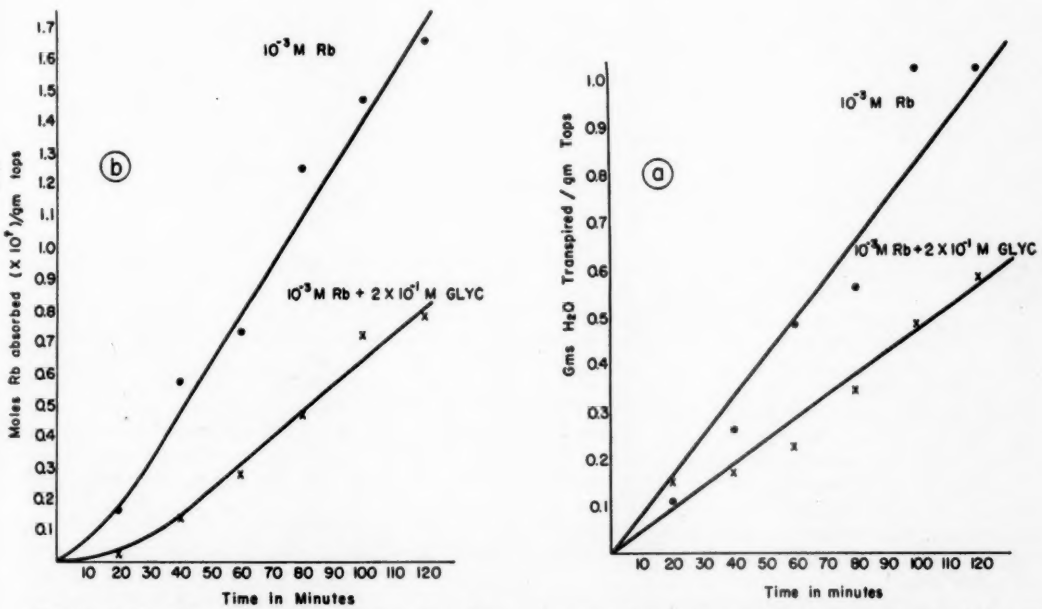
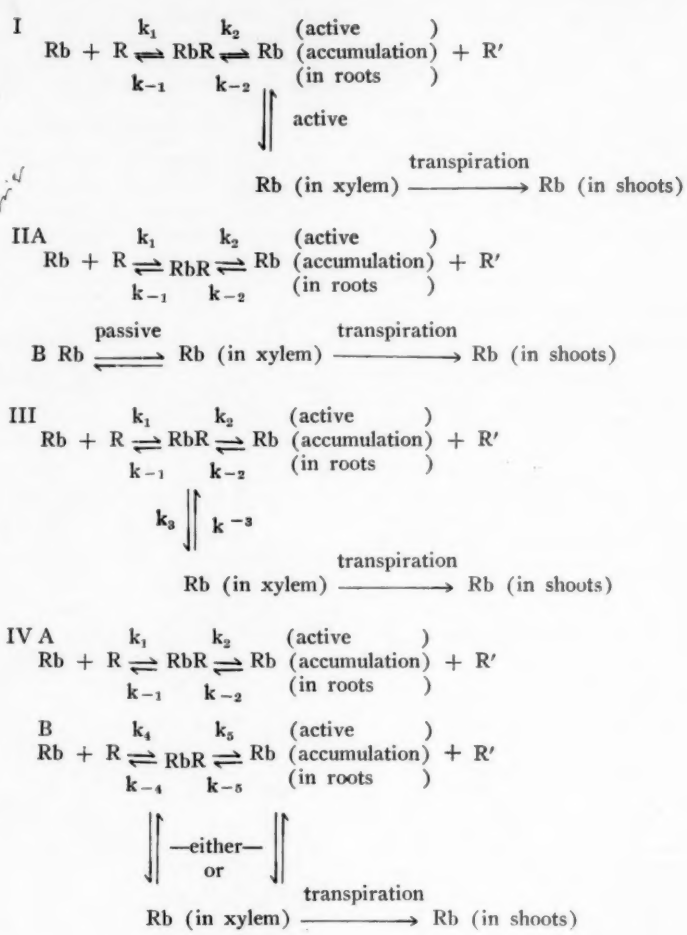

b
10⁻³ M Rb
10⁻³ M Rb + 2 X 10⁻¹ M GLYC
Moles Rb absorbed (X 10⁶)/gm tops
Time in Minutes
a
10⁻³ M Rb
10⁻³ M Rb + 2 X 10⁻¹ M GLYC
Gms H₂O Transpired / gm Tops
Time in minutes

FIG. 6a & 6b. The effect of 2×10^{-1} M glycerine on the rates of water loss and ion accumulation by 7-day-old barley plants. a) rate of water loss by evaporation, b) rate of Rb accumulated in shoots.

one of the processes that is known to occur, it appears in all sets of equations. The equations are shown below:


I
Rb + R ⇌ RbR ⇌ Rb (active accumulation in roots) + R′
k1 k2 k−1 k−2
active
Rb (in xylem) → Rb (in shoots)
transpiration
IIA
Rb + R ⇌ RbR ⇌ Rb (active accumulation in roots) + R′
k1 k2 k−1 k−2
B Rb ⇌ Rb (in xylem) → Rb (in shoots)
passive
transpiration
III
Rb + R ⇌ RbR ⇌ Rb (active accumulation in roots) + R′
k1 k2 k−1 k−2
k3 k−3
Rb (in xylem) → Rb (in shoots)
transpiration
IV A
Rb + R ⇌ RbR ⇌ Rb (active accumulation in roots) + R′
k1 k2 k−1 k−2
B
Rb + R ⇌ RbR ⇌ Rb (active accumulation in roots) + R′
k4 k5 k−4 k−5
—either—
or
Rb (in xylem) → Rb (in shoots)
transpiration

Each set of equations will be considered in detail. Equation I states that all ions reaching the shoots go through the active carrier accumulation process in the roots. When written this way, the concentration of ion actively accumulated in the roots determines the shoot accumulation of the ion. This description of ion transport is inconsistent with the data of Wiebe and Kramer (28) and Epstein (7). Wiebe and Kramer (28) showed that the region of most active accumulation in the root did not necessarily coincide with the region of maximum transport. Epstein (7) showed that when roots were taken out of the outside sulfate solution, transport to shoots was reduced to a very low level.

To test equation I, an experiment with Rb similar to the sulfate experiment of Epstein (7) gave results recorded in table III. At the end of the dark period, the shoots of the plants in both treatments 1 and 2 contained 0.12×10^{-7} moles of radioactive Rb per gram and treatment 3 none. At the start of the light period the roots in treatment 1 and 2 actually contained 1.8×10^{-7} moles of radioactive Rb per gram while those of treatment 3 contained none. At the end of the light period, the shoots in treatment 2 contained 0.20×10^{-7} moles per gram, a net increase of 0.08×10^{-7} moles and treatment 3 a net increase of 0.31×10^{-7} moles per gram. The root content of radioactive Rb at the end of the light period of treatment 3 was the same as at the end of the dark period of treatments 1 and 2. Thus treatment 2 with more than adequate root content of radioactive Rb to supply all the shoot requirements and in fact with a higher root content during most of the light period than treatment 3, only accumulated in the shoots one-

TABLE III

EFFECT OF TREATMENT OF BARLEY PLANTS WITH 1×10^{-4} M RbCl ON APPEARANCE OF RUBIDIUM IN SHOOTS

TREATMENT NO.	DARK TREATMENT	LIGHT TREATMENT	RADIOACTIVE RUBIDIUM IN SHOOTS	
			TOTAL	NET
			Moles ($\times 10^7$)/g	
1	60 min radioactive Rb plus 60 min H_2O	Harvested	0.12	...
2	60 min radioactive Rb plus 60 min H_2O	60 min inactive Rb plus 60 min H_2O	0.20	0.08
3	60 min inactive Rb plus 60 min H_2O	60 min radioactive Rb plus 60 min H_2O	0.31	0.31

fourth as much radioactive Rb as treatment 3. Therefore, the root concentration of accumulated rubidium is not the primary source of rubidium to shoots as predicted from equation I.

Epstein (7) and Hylmo (19) suggested an alternative which can be described by equations II A and B although both authors recognized that all transport did not necessarily occur this way. These equations state that two parallel processes occur, active accumulation by the roots and passive movement of ions to the xylen where they are then moved to shoots by the transpiration stream.

The results with the inhibitors, DNP, K^+, H^+, sodium azide, and methylene blue suggest that equations II A and B likewise do not adequately describe the system. The effect of DNP, usually an uncoupler but non-respiratory type inhibitor, and azide, a respiratory inhibitor, was the same at all concentrations of rubidium, suggesting active accumulation as dominant at all concentrations. Methylene blue, another respiratory inhibitor, only affected uptake at the low concentrations and not at the high. Results of Brouwer (3) and Russell, et al (26, 27) also suggest that equations II A and B are not fully descriptive of the system.

Another alternative to equation I that is not dependent on the total concentration of active root accumulated ions is equation III. Equation III states that the same active intermediate is involved in ion accumulation by roots and shoots. In contrast to equation I, shoot accumulation is not functional on the total accumulation in the root. The shoot accumulating pathway can be considered as a competing reaction with roots for RbR.

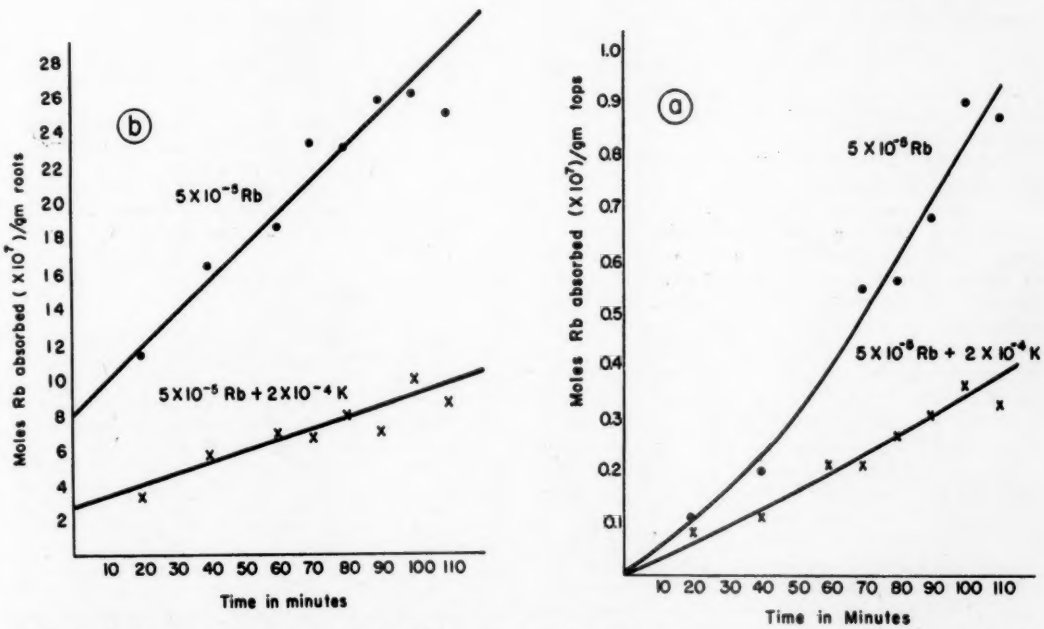


FIG. 7a & 7b. The effect of 2×10^{-4} M K in a 5×10^{-5} M Rb solution on the rate of ion accumulation in shoots and roots by 7-day-old barley plants. a) shoots, b) roots.

Since RbR in this model is a constant in a steady state system, any change in RbR should have a proportionate effect on both active accumulation in the roots and in the shoots. A competitive inhibitor like potassium, which does not change either the apparent reaction rate constants or the concentration of carrier, but does change RbR concentration, should change root and shoot ion accumulation proportionately. Figures 7a and 7b show the effect of 2×10^{-4} M potassium on the accumulation of Rb by shoots and roots at 5×10^{-5} M Rb. The addition of a competing ion has a proportionate effect on Rb ion accumulation in shoots and roots. Thus, after steady state is reached, the change in slope in figures 7a and 7b is approximately 3.1.

When the apparent dissociation constant, Km, for the active intermediate that predominates at lower Rb concentrations is calculated from the data in figure 2a and 2b, or other individual experiments, the values are always the same order of magnitude. The Km for roots and shoots is approximately 4×10^{-5}. This is in marked contrast to the differences in root-shoot distribution found by Russell and Martin (25) for phosphorus at lower concentrations and longer uptake periods.

The similarity of apparent dissociation constants for roots and shoots is consistent with equation III but not equation II. Furthermore, the observation that shoots as well as roots show two sites of ion uptake indicates a close relationship between shoot and root accumulation. Again this is consistent with equation III.

The data observed in these experiments are consistent with the model discussed in equation III. The effect of inhibitors may differ because of differing effects on the active intermediate, RbR, or the two metabolic rate constants, k_2 and k_3. The inhibitors, of course, may also change the concentration of the metabolic carrier.

Equation III represents ion accumulation to shoots as a series of reactions. The rate limiting step in the sequence may vary with the experimental conditions. The data presented suggest that under the experimental conditions used the biological constants of the active carrier mechanism determine the concentration in the xylem while the transpiration rate determines the rate of movement to the shoots.

Equation IV also fits all the data since it places no restrictions on the system. It represents two parallel active accumulation mechanisms, one leading primarily to root accumulation and one leading to shoot accumulation. Equation IV B can be represented either as in equation I or equation III. Not only the rate constants of equation IV B but even the carriers can be represented as different from equation IV A. However, equation IV A and IV B may involve the identical carriers and rate constants but occur in different locations in the root. Since equation III places more restrictions on the system it is more attractive to use until proven inconsistent with further experimental data.

Shoot accumulation reflects both active metabolic processes and transpiration without being directly dependent on previously accumulated ions. The ions that reach the shoots have gone through an active metabolic process.

Summary

Short time steady-state accumulation studies of radioactive rubidium by roots and shoots of 7-day-old barley plants indicate the relationship between active and passive processes in ion accumulation by shoots. If the carrier mechanism is used to describe the active process, the observed data are consistent with two competing active processes for an active intermediate, one leading to root accumulation and the other to accumulation in the shoot. The data are also consistent with two parallel active processes, one leading to root accumulation and the other to shoot accumulation.

Shoot accumulation showed two concentration functions, one that predominated at high Rb concentration and another that predominated at lower Rb concentrations. These corresponded with the two concentration functions for Rb uptake by roots.

Azide, methylene blue, DNP, potassium, and H ion were all shown to inhibit active rubidium accumulation but not always in the same way. The action of these inhibitors and the apparent dissociation constant, Km, of the rubidium carrier combination were similar for roots and shoots.

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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON UTILIZATION OF LABELED ACETATE BY BEAN LEAF & STEM TISSUES [1, 2, 3]

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An increase of $C^{14}O_2$ production from acetate-1-C^{14} and acetate-2-C^{14} in corn roots by 2,4-D treatment has been reported; the percentage increase was larger from the methyl carbon than from the carboxyl carbon (4). Using root tissues it was found that 2,4-D caused an increase in the amount of glucose catabolized via the pentose phosphate pathway in root tissues (3). When bean stem tissue was used in a radio-respirometric experiment, 2,4-D caused a decrease in the amount of glucose catabolized via the pentose phosphate pathway (1). Since acetate occupies a key position in the metabolic pathways of amino acids, carbohydrates, and fatty acids, the effect of 2,4-D on acetate utilization in other plant tissues was investigated.

MATERIALS & METHODS

Bean plants (*Phaseolus vulgaris* var. Black Valentine) were grown in the greenhouse in metal flats containing Newberg sandy loam. The plants were treated when the primary leaves were almost fully expanded and the terminal buds were still very small. A 95 % ethanol solution containing 0.5 % 2,4-D acid and 0.2 % Tween-20 was used for treatment. A microsyringe was used for applying 2,4-D solution. In the experiments with stem tissue, the total amount of 2,4-D was applied to each plant along the midrib of one primary leaf, while in the experiments with leaf tissue, the amount of 2,4-D was equally divided and applied to both primary leaves. After 7 days of treatment, either the first internodes or leaves were harvested. Two 2-cm sections were cut from each internode. Each leaf was cut with a cork borer into four disks, 1 cm in diameter. Approximately one gram of fresh stem sections or 30 to 32 leaf disks (300–400 mg) were placed in incubation flasks containing 10 ml of 0.01 M potassium phosphate buffer, pH 5.2, and 0.5 mg sodium acetate-C^{14}. The flasks were shaken continuously for 6 hours at 26° C and the $C^{14}O_2$ released in respiration was collected periodically in NaOH solution. Detailed procedure was given in a previous paper (1). In the case of leaf tissues, the incubation was done in complete darkness by wrapping the flask with light proof paper, while with stem sections the flasks were not covered and thus were exposed to normal laboratory lighting from the ceiling. At the end of incubation, the tissues were removed from the medium, rinsed with water, and put into boiling 80 % alkaline alcohol (0.001 N NaOH) for 3 minutes to stop the reaction. The tissues were finely ground in a mortar, and the homogenate was made up to 25 ml. The insoluble residue was filtered out and washed thoroughly with 80 % alcohol. The $C^{14}O_2$ from respiration was precipitated as $BaCO_3$. The radioactivities of the $BaCO_3$, the alcohol extract of the tissues and the alcohol insoluble residue were determined with a thin mica window G-M counter (1.8 mg/cm^2). Occasionally, the radioactivity of the medium after incubation was also determined in order to estimate the total recovery of radioactivity in each run.

Aliquots of the alcohol extract were acidified with dilute acetic acid and evaporated to dryness in order to remove free radioactive acetate and other volatile acids. The difference in the radioactivity of alcohol extract before and after acidification was used as a measure of the free radioactive acetate. After removal of the volatile acids, the alcohol extract was subjected to isooctane extraction. The radioactivity removed by isooctane extraction was designated as crude lipids.

Both sodium acetate-1-C^{14} and sodium acetate-2-C^{14} were purchased from Volk Radiochemical Co. All labeled substrates were adjusted to 2 μc per mg of sodium acetate prior to use.

RESULTS & DISCUSSION

Effect of 2,4-D on the uptake and utilization of labeled acetate by bean stem and leaf tissues are shown in table I. The data are expressed as per cent distribution of C^{14} from absorbed acetate in various fractions. The actual amounts of acetate absorbed, metabolized, and incorporated per 1 gram fresh tissues during a 6-hour experimental period are presented in parentheses below the per cent values. The average total recovery of isotope from 16 runs each in the experiment with the stem tissue was 97.1 % for acetate-1-C^{14} and 96.2 % for acetate-2-C^{14}, while with the leaf tissue it was not determined. In view

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[3] Technical Paper no. 1350, Oregon Agricultural Experiment Station.

of severe toxic effect on the leaf when a higher dosage of 2,4-D was used, no treatment higher than 50 μg per plant is included. All plants which received 50 μg or less per plant were healthy 7 days after treatment.

Acetate uptake by bean stem from 2,4-D treated plants showed a twofold increase over the stem sections from the untreated control. No difference was evident on the substrate uptake of stem sections from plants receiving 50, 100, or 150 μg 2,4-D per plant. With the exception of 10 μg per plant treatment, an increase of 30 % in the amount of acetate taken up by the leaf tissues from 2,4-D treated plants was also observed.

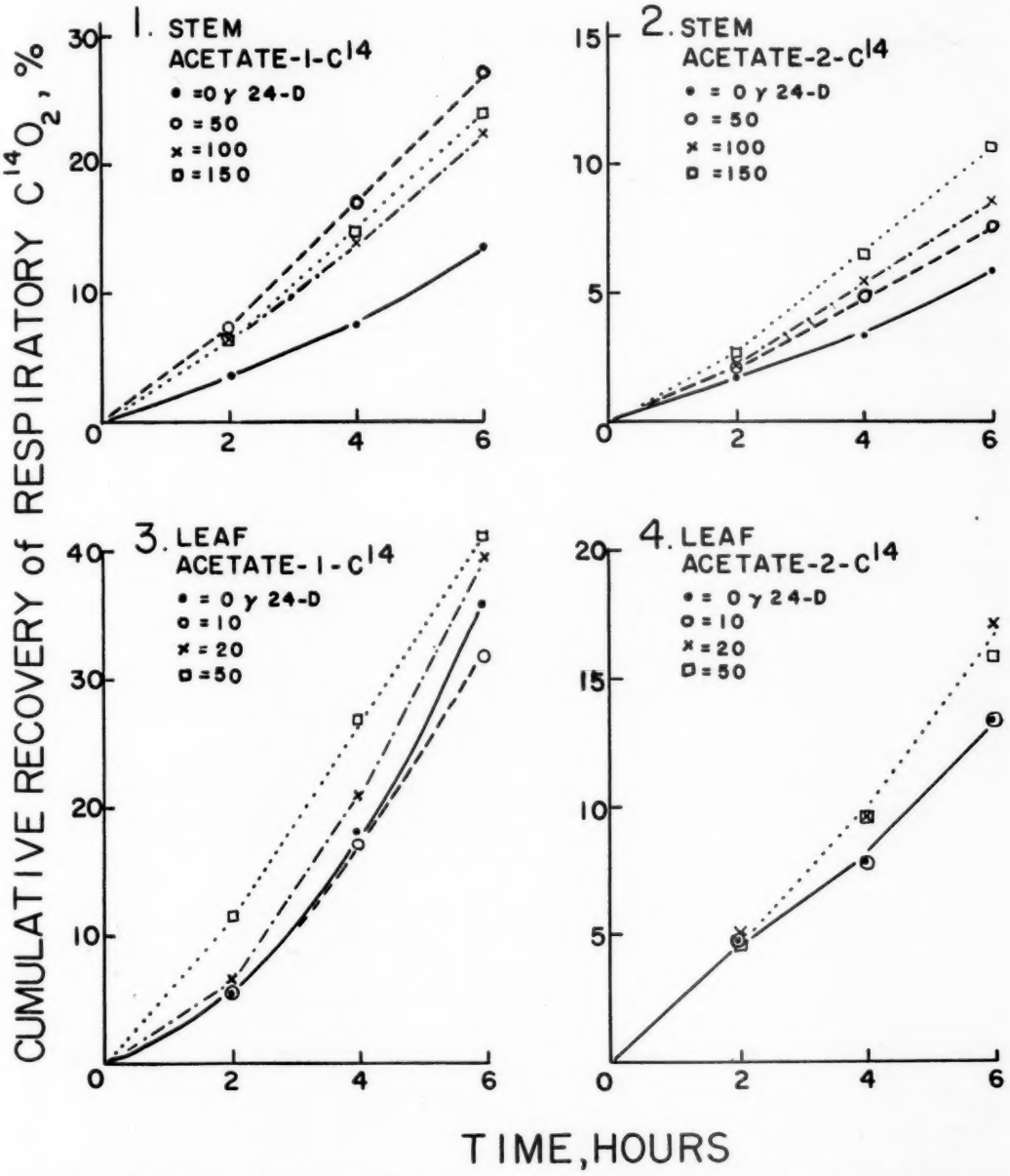

FIG. 1–4. Time course plots of radiochemical recoveries in respiratory CO_2 from C^{14} labeled acetate by bean stem and leaf tissues from control and 2,4-D treated plants.

Percentage radiochemical yield of $C^{14}O_2$ from acetate-1-C^{14} and acetate-2-C^{14} was increased in 2,4-D treated bean stem tissues with no significant differences among three levels of treatment. The increase was greater from the carboxyl carbon than from the methyl carbon. In the bean leaf tissues the data revealed no significant change on the percentage distribution of C–1 and C–2 carbons in the respiratory CO_2 by 2,4-D treatment. However, actual amount of catabolic oxidation of acetate was increased and this increase approximately corresponded to the magnitude of increase for substrate uptake. There is evidence now that light affects CO_2 evolution and other metabolic pathways. The differences observed between leaf and stem tissues attributed to the effect of 2,4-D may actually be due to light in these experiments. The average values were 13.4±1.3 % and 5.8±0.5 % from acetate-1-C^{14} and acetate-2-C^{14}, respectively, in the control stem tissues and were 36.0±2.8 % and 13.3±1.5 % in the control leaf tissues. The ratio of C–1/C–2 calculated from $C^{14}O_2$ data was 2.3 for stem tissues and 2.7 for leaf tissues which agreed reasonably well with the results reported for peanut cotyledons (5) and corn roots (4). Treatment with 2,4-D at the dosages tested did not significantly alter the C–1/C–2 ratio either in the stem (2.3–3.5) or in the leaf tissue (2.3–2.6).

During this 6-hour incubation period the respiratory $C^{14}O_2$ recovery in stem tissues from either labeled acetate disclosed an almost linear increase with time (figs 1 & 2). 2,4-D Treatment approximately doubled the percentage incorporation for C–1 carbon while only a small increase was evident for C–2 carbon. Increasing the amount of treatment from 50 µg to 150 µg per plant did not produce an additional effect for C–1. The percent incorporation of C–2 carbon of acetate into respiratory CO_2 appeared to be increased with increasing 2,4-D dosage. This effect was more noticeable at later periods than at early periods. Since the experiment was repeated only once for higher dosage treatments (100 & 150 µg/plant), the increasing trend of CO_2 production for C–2 carbon due to increasing 2,4-D dosage is not statistically significant. Figures 3 and 4 illustrate the cumulative recovery of C^{14} in respiratory CO_2 by bean leaf tissues. It is obvious from these curves that the rate of C–1 carbon incorporation increased as incubation time progressed. This trend was not observed for C–2 carbon. Higher dosages (20 µg & 50 µg/plant) of 2,4-D treatment definitely increased both C–1 and C–2 incorporation into CO_2 but in the low dosage of 2,4-D (10 µg) its influence was not noticeable.

Percentage incorporation of C–1 and C–2 carbons

TABLE I

EFFECTS OF 2,4-D TREATMENT ON ABSORPTION & METABOLISM OF ACETATE-C^{14} IN BEAN STEM & LEAF TISSUES FROM PLANTS WHICH RECEIVED VARIOUS DOSAGES OF 2,4-D

			% DISTRIBUTION OF C^{14} FROM ABSORBED ACETATE IN PLANT FRACTIONS					
	2,4-D DOSAGE µg/PLANT	TISSUE USED	ABSORPTION OF ACETATE/gm FR TISSUE 6 hr		RESPIRATORY CO_2		ETOH INSOLUBLE RESIDUE	
			1-C^{14} µg	2-C^{14} µg	1-C^{14}	2-C^{14}	1-C^{14}	2-C^{14}
A.	Control (6)*	Stem	88	92	13.4±1.3** (11.8)***	5.8±0.5 (5.3)	23.3±7.6 (20.5)	32.4±3.9 (29.8)
B.	50 (6)	Stem	176	213	27.0±2.3 (47.5)	7.7±1.4 (16.4)	10.0±2.6 (17.6)	19.1±3.6 (40.7)
C.	100 (2)	Stem	215	156	22.2±0.3 (47.7)	8.6±0.5 (13.4)	11.0±0.1 (23.7)	19.9±1.3 (31.0)
D.	150 (2)	Stem	210	166	23.8±0.2 (50.0)	10.6±0.4 (17.6)	11.4±0.6 (23.9)	21.9±0.5 (36.4)
E.	Control (3)	Leaf	326	309	36.0±2.8 (117.4)	13.3±1.5 (41.1)	11.3±1.5 (36.8)	21.4±6.3 (66.1)
F.	10 (3)	Leaf	330	336	32.4±0.6 (106.9)	13.2±2.4 (44.4)	21.5±2.5 (71.0)	24.9±6.9 (83.7)
G.	20 (3)	Leaf	413	413	39.6±1.2 (163.5)	17.1±3.2 (70.6)	12.8±3.0 (52.9)	20.1±6.3 (83.0)
H.	50 (3)	Leaf	412	418	41.2±2.0 (169.7)	15.8±2.2 (66.0)	12.1±4.6 (49.9)	19.1±7.1 (79.8)

* Denotes number of separate runs.

** Mean and standard deviation.

*** Mean (µg sodium acetate/gm fr wt tissue/6 hr).

of acetate into the alcohol insoluble residue was greatly reduced in the stem tissues from 2,4-D treated bean plants. The degree of reduction was not further enhanced by increasing 2,4-D dosage. On the other hand, either no change or an increase in such incorporation was found in the leaf tissues. This observation would substantiate the finding by others that the influence of 2,4-D on plants depends largely upon the type of tissue used (1, 3). If we were considering the actual amount of acetate metabolized per unit weight of fresh tissues, 2,4-D treatment produced a fourfold increase of catabolic oxidation for C–1 and approximately 2.5- to 3-fold increase for C–2 carbon in the stem, with no change for synthesis. Therefore, the reduction of percentage incorporation of isotopic carbon into the alcohol insoluble residue is due to an increase of catabolic function with no change in anabolic function. In the leaf tissues, low dosage (10 μg) of 2,4-D significantly increased the anabolic activity with no noticeable change of catabolic oxidation, while at higher dosages, both anabolic and catabolic activities were increased.

A greater increase of free acetate was found in 2,4-D treated stem over the untreated controls, while no change was observed in the leaves. Such increase could be due to an increase in substrate uptake with no (or slight) increase in metabolic activity.

The actual participation of C–2 carbons of acetate in the synthesis of lipid was affected only by higher dosage of 2,4-D (150 μg) in the stem, while no significant differences were found either with C–1 or with C–2 carbon in the leaf. The ratio of C–2/C–1 calculated from the result of percentage carbon participation in lipids' synthesis indicated that at 50 and 100 μg levels there was no change from the control; however, at 150 μg level the participation of C–2 in lipids' synthesis was increased. The formation of long chain fatty acids by means of the multiple β-condensation of acetate has been demonstrated in developing flax fruits (2). Stumpf and Barber (7) also provided experimental evidence showing synthesis of fatty acids by multiple β-condensation of C_2 units in avocado fruit mesocarp. If multiple β-condensation of C_2 units is the principal pathway of synthesizing fatty acids, the isotope incorporation into lipids with acetate-1-C^{14} and acetate-2-C^{14} (C–2/C–1 ratio) should be near unity. Data in table I showed that C–2/C–1 ratio in the leaf lipids is 1, while in stem lipids is 1.5. It is reasonable to assume that this difference could be due to the participation of other pathway(s). Utilization of the methyl group of methionine in the formation of fatty acids in microorganisms has been demonstrated (6, 8). It is possible that the incorporation of methyl carbon of acetate into fatty acids may also arise from the same pathway which may involve either direct or indirect utilization of methyl carbon as a C_1 fragment. Therefore, it is concluded that 2,4-D has no effect on the

TABLE I CONT.

	% DISTRIBUTION OF C^{14} FROM ABSORBED ACETATE IN PLANT FRACTIONS						
	EtOH EXTRACT OF TISSUE						
	Total		VOLATILE ACID		ISOOCTANE SOLUBLE		
	1-C^{14}	2-C^{14}	1-C^{14}	2-C^{14}	1-C^{14}	2-C^{14}	C_2/C_1
A.	63.3±8.4 (55.7)	61.9±3.9 (56.9)	27.5 (24.2)	26.3 (24.2)	6.5 (5.7)	9.6 (8.8)	1.5
B.	63.0±2.6 (110.9)	73.2±5.0 (155.9)	44.0 (77.4)	39.8 (84.8)	2.8 (4.9)	4.6 (9.8)	1.6
C.	66.9±0.5 (143.8)	71.5±0.8 (111.5)	37.8 (81.3)	31.6 (49.3)	2.9 (6.2)	5.5 (8.6)	1.9
D.	64.9±0.4 (136.3)	67.5±0 (112.1)	35.3 (74.1)	36.1 (59.9)	2.4 (5.0)	11.0 (18.3)	4.6
E.	52.7±4.7 (171.8)	65.4±5.2 (202.1)	8.7 (28.4)	11.8 (36.5)	1.1 (3.6)	1.1 (3.4)	1.0
F.	46.1±2.9 (152.1)	61.8±4.5 (207.6)	12.0 (39.6)	9.4 (31.6)	1.1 (3.6)	1.7 (5.7)	1.5
G.	47.6±1.9 (196.6)	62.8±3.0 (259.4)	8.0 (33.0)	7.4 (30.6)	0.8 (3.3)	1.0 (4.1)	1.3
H.	46.7±4.6 (192.4)	65.2±5.0 (272.5)	7.5 (30.9)	8.3 (34.7)	0.8 (3.3)	1.2 (5.0)	1.5

TABLE II

% DISTRIBUTION OF RADIOACTIVITY AMONG METABOLIC END PRODUCTS OF BEAN STEM & LEAF TISSUES INCUBATED IN ACETATE-C^{14} SOLUTION

2,4-D TREATMENT	TISSUES	C-1			C-2		
		E.R.[1]	T.S.[2]	E.S.[3]	E.R.[1]	T.S.[2]	E.S.[3]
Control	Stem	31	54	15	12	68	20
50 μg	Stem	68	25	7	25	61	14
100 μg	Stem	62	30	8	25	59	16
150 μg	Stem	63	30	7	25	50	25
Control	Leaf	75	23	2	37	60	3
10 μg	Leaf	59	39	2	33	63	4
20 μg	Leaf	74	24	2	45	53	2
50 μg	Leaf	76	22	2	44	53	3

[1] Energy release (CO_2)

[2] Tissue synthesis (residual)

[3] Energy storage (lipid synthesis)

formation of fatty acids by multiple β-condensation of acetate and probably stimulates the synthesis of fatty acids via other pathway(s). Further investigation of a possible one-carbon addition pathway in the formation of lipids in the higher plants may be of value in understanding not only the biosynthesis of lipids but also the action of 2,4-D on lipid synthesis.

It is reasonable to assume that the 80 % ethanol extract (excluding lipids) contains only free acetate and other intermediate metabolites which will eventually incorporate into these three fractions (CO_2, catabolic end product; alcohol insoluble residue, anabolic products for the synthesis of cell wall materials; & lipids, energy storage). Thus, the specific influence of 2,4-D on the relative utilization of C-1 and C-2 carbons in these three functions which were calculated directly from the incorporation data on metabolic end products by bean stem and leaf tissues, are clearly shown in table II.

SUMMARY

In vitro studies on the utilization of acetate by leaf and stem tissues were carried out using carbon labeled acetates. The stem tissues from bean plants receiving various amounts of 2,4-D showed a two-fold increase in substrate absorption, a three- to four-fold increase in catabolic function as indicated by radiochemical yield of respiratory $C^{14}O_2$, and no change in synthetic function as indicated from isotope incorporation into alcohol insoluble residue. Free acetate was significantly increased in 2,4-D treated stem tissues that could result from an increase of substrate uptake. The incorporation of acetate into lipids by multiple β-condensation was not affected in 2,4-D treated stem tissues while by other pathway(s) it was increased.

The effects of 2,4-D on both catabolic and synthetic functions of acetate utilization in bean leaf were slight and dependent to the dosage used. At low dosage, a decrease of catabolic function and an increase of synthetic function was observed. At higher dosages of 2,4-D, a similar increase of catabolic and anabolic activities was evident.

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PRODUCTION OF YELLOW STRAPLEAF OF CHRYSANTHEMUM & SIMILAR DISORDERS BY AMINO ACID TREATMENT[1, 2]

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An apparent physiological disease, Yellow Strapleaf (YSL) of *Chrysanthemum morifolium* Ram., was described by Jackson and Woltz (1) in 1959. The disease has been observed in Florida plantings during the last 6 years and has been noted in Massachusetts[3]. Symptoms include chlorosis of new growth, green netting of leaves, and narrow strap-shaped leaves that hook at the distal ends (fig 1A). The condition may persist for 2 to 8 weeks causing mild to severe stunting of plants and has been found in 20 commercial chrysanthemum varieties. YSL has not been corrected by the application of inorganic nutrient elements or linked directly with any fungal, bacterial, or viral pathogen. Chemical analyses of plant and soil have not indicated any significant differences in content of inorganic nutrients. Higher soil pH was usually associated with the disorder.

Woltz and Jackson (6) presented results of experiments that linked frenching of tobacco (*Nicotiana tabacum* L.) and YSL of chrysanthemum as being apparently caused by similar soil conditions and resulting in similar syndromes. Both disorders are favored by relatively high soil pH, periods of high soil moisture levels, and high soil temperatures. The symptoms displayed with the diseases (1, 5) have three common features: A: green netting or reticular chlorosis of leaves, B: narrow, strap-shaped leaves, and C: growth retardation. Tobacco and chrysanthemum plants growing together in containers of soil that had produced YSL in the field developed symptoms of the respective diseases of frenching and YSL. Following the information set forth by Steinberg (5) that isoleucine and certain other amino acids were effective in producing frenching, DL-isoleucine with DL-alloisoleucine was applied to the root zone of chrysanthemum plants. This amino acid treatment uniformly and quickly produced YSL symptoms with plants growing in solution culture, steamed soil, methyl bromide-treated soil, untreated soil, quartz sand and expanded volcanic glass (perlite). Six chrysanthemum varieties of varying degrees of susceptibility to YSL were treated with isoleucine in this experiment. YSL symptoms developed in all varieties with severity approximately in the order of observed field susceptibility.

This report presents data on the effects of certain specific amino acids on the growth of chrysanthemums.

METHODS

Rooted Iceberg variety chrysanthemum cuttings were planted March 11, 1960, in 4-inch plastic flower pots containing methyl bromide-treated sandy soil. One cutting in each of three replicate pots represented a treatment. Plants were fertilized weekly with a solution containing essential nutrient elements.

Amino acid and ammonium nitrate solutions of standard volume were applied to the soil surface around the base of each plant. Amounts applied, expressed in millimoles (mmoles) per plant, were the following: DL-alloisoleucine, 0.0125, 0.05, 0.2, and 0.8; D-alloisoleucine, 0.0125, 0.05, 0.2, and 0.8; L-isoleucine, 0.8 and 3.2; D-isoleucine, 0.8; L-leucine, 0.8, 3.2, and 6.4; D-leucine, 0.8; L-methionine, 0.8 and 3.2; D-methionine 0.8 and 3.2 mmoles; and controls consisting of varied amounts of ammonium nitrate to equal the amount of nitrogen supplied by each amino acid treatment. The L-isoleucine used was an allo-free grade. Other amino acid isomers were of the highest grade available from reputable biochemical suppliers.

The selection of dosage levels of the amino acid isomers was based on the results of preliminary experiments showing the relative effectiveness of the isomers as well as the available supply of the various isomers.

Half of the amount of amino acids intended for each plant was applied March 29 immediately after removal of the terminal growing points. The root systems were well established at this time. Three days later the remaining quantities of amino acids were applied. Heights of the uppermost new shoots were measured April 6, 14, and May 30, since growth retardation was considered the most significant, measurable index of YSL or other effects of amino acids. The plants were observed daily to note changes in leaf shape and the development of chlorotic patterns.

RESULTS

Mean heights of upper new shoots are given in figure 2. The heights of the new shoots at each of the dates are expressed on a relative basis, setting the

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[2] Florida Agricultural Experiment Station Journal Series no. 1113.

[3] Personal communication from E. C. Gasiorkiewicz, Sept. 30, 1959.

FIG. 1. A: The chrysanthemum plant on the right is deformed and stunted by yellow strapleaf; the plant on the left, same age, is normal. Both plants are field-grown Iceberg variety. B: The plant on the left received 0.8 mmoles of DL-alloisoleucine producing yellow strapleaf symptoms. Center plant is a control. The plant on the right received 0.8 mmoles of D-methionine producing symptoms of methionine toxicity (methionosis). Plants were photographed 5 weeks after treatment.

control means as 100. The same shoot on each plant was measured on each of the three dates. Growth increments were not used in the calculations. A positive slope between points indicates a tendency for recovery from the growth inhibiting effects of the amino acids. Negative or no slope indicates a failure to recover. The growth rate of plants recovering from the inhibitory effects of amino acids in some cases exceeded the growth rate of the control plants. For height comparisons at any given date of measurement, the LSD at the 5% level is shown as 100 less the asterisk value. Any value below the asterisk differs significantly from the control.

In figure 2A the growth data for the four D-alloisoleucine levels are averaged and these mean values plotted since none of the individual levels gave growth values significantly different from the control and since there was no visible trend in their effect. Also plotted in figure 2A are the growth values for the individual levels of DL-alloisoleucine. Plants receiving 0.0125 to 0.8 mmoles of D-alloisoleucine exhibited no visible symptoms, whereas all treatments with DL-alloisoleucine produced YSL symptoms. Plants receiving the lowest amount of DL-alloisoleucine recovered from significant growth repression by the second measurement. Plants receiving the intermediate

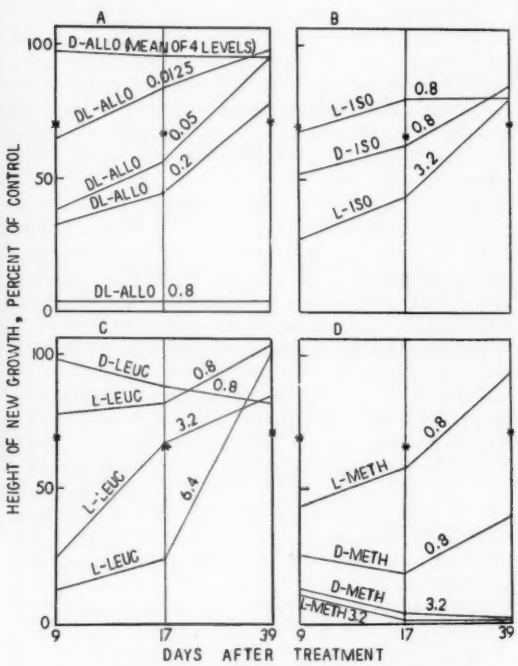

FIG. 2. Elongation of new shoots of chrysanthemum plants receiving indicated amounts in mmoles of isomers of A: alloisoleucine (Allo), B: isoleucine (Iso), C: leucine (Leuc), and D: methionine (Meth) applied to the root zone. Points below asterisks (LSD, 5% level) differ significantly from controls for a given date of measurement.

amounts of 0.05 and 0.2 mmoles had recovered from significant repression by the third measurement. The greatest amount applied (0.8 mmoles) prevented any relative growth recovery (fig 1B).

Both D- and L-isoleucine produced YSL symptoms. The D isomer at the 0.8 mmoles level repressed growth both at the first and second dates (fig 2B) while the L isomer at the same level was not effective beyond the first date. L-isoleucine at 3.2 mmoles per plant repressed growth at the first and second dates of measurement; by the third date, however, the relative growth measurements indicated no significant difference.

D-leucine did not produce YSL symptoms at 0.8 mmoles while L-leucine at the same level produced slight, transient symptoms. Neither D- nor L-leucine significantly suppressed growth at the 0.8 mmoles level (fig 2C), although the L isomer had some effect in this direction. L-leucine at the 3.2 mmoles level repressed growth only at the first measurement. At the 6.4 mmoles level, growth was repressed at the first and second dates; by the third date, however, there was no significant difference.

D- and L-methionine both caused new leaves to become elongated in shape with entire margins and prominent midribs. Leaves developing later were round in shape. The plant with methionine toxicity in figure 1B had not developed final symptoms at the time of the photograph. The symptoms differ obviously, however, from those of YSL.

L-methionine at the 0.8 mmoles level (fig 2D) repressed growth at the first and second dates. The D-isomer at the same level was persistent in its effect through three dates. The difference in height between plants receiving D- and L- methionine at the time of the third measurement was quite pronounced indicating the L isomer was decreasing more rapidly in effectiveness with the passage of time. The results at the 3.2 mmoles level for both isomers were quite similar and the effect in growth suppression continued very strong at the third measurement date.

Undimensional paper chromatograms of leaves naturally affected with YSL showed at least 100% greater total free amino acid content than normal leaves. Soils extracted with water and various reagents were found to contain free amino acids of leucine and isoleucine Rf values as well as others.

Foliar Uptake. Single plants of Iceberg variety chrysanthemum growing in methyl bromide-treated sandy soil in 4-inch plastic flower pots were used to determine whether DL-alloisoleucine taken up through the foliage would produce YSL symptoms. Pots were inclined and a leaf on one of the three shoots of each plant was immersed for 72 hours in A: 100 ml of deionized water (control) and B: 100 ml of 0.03 M DL-alloisoleucine. The controls exhibited no abnormalities while the younger leaves on the shoots above the treated leaves developed a green netting with chlorotic interveinal areas. This condition is characteristic of the first stages of YSL produced either naturally in the field or by the administration of isomers of isoleucine around the roots of the plant. Spraying the foliage of chrysanthemum plants with 0.075 M DL-alloisoleucine had no effect. By decapitating a shoot and retaining a 0.2 M solution of DL-alloisoleucine around the cut portion of the stem for 5 days it was possible to produce the same early YSL symptoms described above.

Experiments With Other Plants. The possibility that naturally occurring disorders similar to YSL might be caused by amino acids in the soil was investigated. An assortment of genera representing 11 plant families was planted in containers of methyl bromide-treated soil in the greenhouse. Planting dates were selected so that the plants would be about the same size, 6 to 12 centimeters high, at the time of treatment. Plants were spaced about one per 60 square centimeters of area. Treatments, replicated three times were: A: DL-isoleucine containing unspecified amounts of DL-alloisoleucine, 1.2 mmoles/100 sq cm; B: L-leucine 1.2 and 4.8 mmoles per 100 square centimeters; C: DL-methionine, 0.6 mmoles/100 sq cm; and D: ammonium nitrate (control), 0.6 mmoles/100 sq cm.

The effects produced in all replications are described in table I, except for L-leucine which had no apparent effect. The symptoms produced by isoleucine are somewhat similar for all plants tested. Affected plants exhibited some or all of the general symptoms that follow: A: green netting of leaves, B: narrowing of leaf or leaflet, and C: abnormal leaf pattern in regard to vein and marginal characteristics (veins frequently were more prominent & leaf margins lost their lobate characteristics). An exception was *Zea mays* (corn) which had an entirely different syndrome as described in table I. Corn was the only grass tested. Grasses as a group may have an entirely different response.

Leaf symptoms resulting from isoleucine treatment of tomato plants were generally similar to the symptoms of tobacco mosaic virus disease of tomatoes. Squash and cucumber treated with isoleucine had leaf symptoms similar to the cucumber mosaic virus disease of these two plants.

Discussion

Data of Putnam and Schmidt (4) together with preliminary, unpublished results of the present authors indicate that naturally-occurring free amino acids are available to plant roots under many soil conditions. It is well established in the literature (5) that plants are capable of taking up amino acids by way of their roots. These considerations coupled with the data in this report on the effect of isomers of leucine, isoleucine, and methionine lead to the suggestion that naturally occurring amino acids may act as growth inhibitors and constitute potential causal agents of abnormal plant growth in the field. YSL of chrysanthemum and frenching of tobacco are like-

TABLE I

SYMPTOMS PRODUCED BY APPLYING DL-ISOLEUCINE PLUS DL-ALLOISOLEUCINE & DL-METHIONINE TO ROOT ZONE OF INDICATED PLANTS

BINOMIAL	COMMON NAME	ISOLEUCINE	METHIONINE
Ambrosia trifida L.	Ragweed	Green netting of leaves	...
Antirrhinum majus L.	Snapdragon	None	None
Beta vulgaris L.	Beet	None	None
Bidens pilosa L.	Bur marigold	Green netting of leaves	...
Brassica oleracea v. gongylodes L.	Kohlrabi	None	...
Callistephus chinensis (L.) Nees	Aster	None	None
Capsicum frutescens L.	Pepper	Green netting of leaves	None
Cucumis sativus L.	Cucumber	Leaves small, chlorotic, very serrate, with prominent veins	...
Cucurbita pepo L.	Squash	Leaves narrow, chlorotic, sharp lobed, with prominent veins	...
Dianthus armeria L.	Grasspink	Green netting of leaves, stunting	None
Lathyrus odoratus L.	Sweet pea	None	None
Limonium sinuatum (L.) Mill.	Statice	None	None
Lycopersicon esculentum Mill.	Tomato	Green netting of leaves, with mottling & puckering; narrow, slightly twisted leaflets	None
Nicotiana tabacum L.	Tobacco	Green netting of leaves, stunted plants	...
Petunia hybrida Vilm.	Petunia	Green netting of leaves	None
Phaseolus vulgaris L.	Bean	Green netting of leaves, narrow leaves, short plants	Narrow leaves, prominent sometimes translucent veins, epinasty of leaf tip, cupping of leaves, short plants
Pisum sativum L.	Pea	None	None
Salvia farinacea Benth.	Blue salvia	Green netting, narrow leaves	...
Tagetes erecta L.	Marigold	Narrow, strap-shaped yellow leaves; loss of marginal indentations	None
Tropaeolum majus L.	Nasturtium	Green netting of leaves	None
Zea mays L.	Corn	Watery, brownish breakdown about middle of leaves, suggestive of calcium deficiency symptoms	None Same as for isoleucine
Zinnia elegans Jacq.	Zinnia	Green netting of leaves; strap-shaped, narrow leaves	None

ly examples of such occurrences. Both represent plant diseases of serious economic consequences in the field. That many plants other than tobacco and chrysanthemum may be affected was demonstrated by the development of definite symptoms in 15 of 22 additional genera tested.

Transient cases of YSL of chrysanthemum were produced by treatment with the appropriate isomers of leucine and isoleucine at the lower levels employed. Transient YSL also occurs in the field. In both instances the same succession of symptom development is generally as follows: A: a green netting of the leaf blade; B: development of partially deformed (loss of part of the normal lobate characteristic) new leaves; and C: the disappearance of the green netting that was present in the older leaves with the concurrent development of normal new leaves.

The symptoms caused by D- and L- methionine are similar to YSL in the early stages; however, as plant growth continues there is no question that the symptoms are different. The name "methionosis" is suggested for the condition produced by excess D- and/or L- methionine in the chrysanthemum plant. Approximately six weeks after treatment with methionine the upper leaves of chrysanthemum plants assume the appearance of Bibb lettuce leaves. The leaves are elongated, have an entire margin, a prominent midrib, and are thin and soft. YSL leaves, in contrast, are very narrow and rather hard in texture, frequently having a hooked tip (fig 1).

The fact that D-isoleucine and D-methionine are more persistent in continuing to produce symptoms than the L isomers may be due to the fact that they are so-called unnatural isomers that are not synthe-

sized into ordinary plant protein. Some organisms contain D amino acid oxidase (2) to transform D-isomers that are not useful and which may, in addition, be toxic; whether chrysanthemums are so supplied is not known. Since D-alloisoleucine was ineffective at all rates and DL-alloisoleucine was highly effective and persistent it seems likely that L-alloisoleucine is the effective portion of the mixture. The literature indicates that L-alloisoleucine has not been found in plant proteins. The high degree of effectiveness and persistence of L-alloisoleucine in causing YSL symptoms suggests that it is not readily incorporated into plant protein.

It is not possible from the data in this report to reach any definite conclusion as to the exact cause of YSL or to the mode of action of amino acids in producing the symptoms. Nevertheless, it is hypothesized from the information at hand that natural YSL may be caused by the growth-modifying effects of certain isomers of leucine and isoleucine taken up by chrysanthemum roots under specific soil conditions. The likelihood of such an hypothesis is strengthened by reports (2, 3) that D-isomers of amino acids including alloisoleucine occur in various natural antibiotic compounds. Obviously, further work is required to clarify the effect of amino acids upon plant growth under natural conditions.

Summary

Symptoms of the yellow strapleaf disease were produced by the application of DL-alloisoleucine, D- and L-isoleucine, and L-leucine individually to the soil surface around the base of chrysanthemum plants. D-alloisoleucine and D-leucine were found to be ineffective in producing these symptoms. D- and L- methionine both caused new leaves to become elongated in shape with entire margins and prominent midribs. Leaves developing later were round in shape. The name "methionosis" is suggested to describe this condition. The height of new growth occurring after the removal of the growing point was significantly reduced only by the isomers that were effective in producing abnormal growth.

The hypothesis is made that yellow strapleaf may be caused by the accumulation of excess amounts of the isomers of isoleucine and leucine in the plant tissue in a free state and that under certain soil conditions such amino acids may be taken up by the roots in amount sufficient to provide the excess.

Plants from 22 different genera of 11 plant families were subjected to treatment with isomers of isoleucine, leucine, and methionine. Isoleucine was most effective, producing symptoms in 15 of 22 genera treated. Leucine was not effective while methionine produced significant effects in two genera.

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STIMULATION OF THE HILL REACTION BY CARBON DIOXIDE[1, 2]

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Hill reactions (i.e., O_2 evolution by illuminated chloroplast preparations) are generally assumed to represent the photochemical splitting of water. For about 20 years the view has prevailed that, in the course of a Hill reaction, photolysis generates an O_2 precursor and a reductant which is capable of reacting directly or indirectly with any of a variety of oxidants. The metabolism of both photolysis products (oxidant & reductant) evidently is enzymatically controlled. The list of oxidizing agents effective in the Hill reaction began chronologically with ferric oxalate and now includes an impressive number of naturally-occurring as well as exotic substances. On the assumption that oxygen evolution and oxidant reduction are separate if not independent processes, there is no compelling a priori reason to believe CO_2 is uniquely involved in the Hill reaction even in a catalytic capacity. However, because of the close analogy between photosynthesis and the various types of Hill reactions, the possible role of carbon dioxide in chloroplast reactions continues to be a subject of concern both theoretically and experimentally, especially so because several reports in the literature suggest that CO_2 at least in some instances may have a catalytic effect on reaction sequences which involve water photolysis.

Franck (7) found an influence of CO_2 on the evolution of O_2 by chloroplasts prepared from tobacco leaf. He studied a Molisch-type reaction in which no Hill oxidant was added to the preparations. Only small amounts of O_2 were evolved but the rate was significantly higher when CO_2 was present. Brown and Franck (3) using $C^{14}O_2$ found that carbon 14 was not taken up under the conditions of these experiments. They concluded that CO_2 could play only a catalytic role.

Kessler (9, 10) observed that the rate of reduction of nitrite by illuminated Ankistrodesmus cells as measured by H_2 uptake was increased in the presence of catalytic amounts of CO_2. Experiments performed with $C^{14}O_2$ showed that the reduction of nitrite with hydrogen was not accompanied by an appreciable fixation of carbon. Thus the effect of CO_2 apparently was indirect.

Boyle (2) reported manometric experiments from which he claimed at least small quantities of carbon dioxide were required for O_2 evolution in the light using triturated spinach leaves. These experiments were repeated by Clendenning and Gorham (6) who found no differences in Hill reaction rate between vessels which contained carbon dioxide and those which did not. Because Boyle's results were not confirmed and besides were incompatible with prevailing theory they have generally been ignored.

Warburg and Krippahl (11, 12) using kohlrabi chloroplast preparations with quinone as the oxidant reported that small amounts of CO_2 increased the rate of the Hill reaction. Similar effects were noted with frozen-dried Chlorella and with quinone-poisoned whole Chlorella cells. Such results are of course reminiscent of the earlier observations by Boyle, even though the experimental material and methods were different.

It is patently desirable to verify the claim that CO_2 can augment the Hill reaction using experimental methods which adhere closely to those of Boyle and of Warburg and Krippahl. We report here the results of such attempts at confirmation. It was found possible to obtain manometric measurements which were in all respects identical with those reported by Boyle although, as will be shown, these results do not support his interpretation that there was a CO_2 effect on the Hill reaction. Manometric results also were obtained which were at least qualitatively similar to the findings of Warburg and Krippahl with kohlrabi chloroplasts. The reactions in which an apparently valid CO_2 effect could be observed also were measured by mass spectrometry which has the important advantage over manometry of relatively unambiguous analytic specificity. Additional experiments were performed with tracer CO_2 and with tracer O_2 which permitted the detection and measurement of simultaneous production and consumption of these gases thus providing detailed information of the nature of the CO_2 effect. This method could reveal the operation of a cyclic regeneration of CO_2 by illuminated kohlrabi chloroplast preparations.

MATERIALS & METHODS

Triturated spinach (*Spinacia oleracea* L.) leaves used in repetitions of Boyle's experiment were pre-

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pared according to the method described by him. (2)

Chloroplasts and chloroplast fragments were obtained from kohlrabi (*Brassica caulorapa* Pasq.) leaves according to the method described by Warburg (11) which consists of grinding the leaves in a waring blender and using differential centrifugation to obtain chloroplast preparations. Microscopic examination of the preparation obtained by this method showed the presence of both whole and fragmented chloroplasts. In both manometric and mass spectrometer experiments the chloroplast preparations were suspended in 0.01 M KCl.

For certain mass spectrometer experiments frozen chloroplast preparations were used. These were thawed immediately before each experiment. Freezing chloroplast preparations suspended in water destroyed their subsequent activity. However suspending chloroplast preparations in 0.5 M sucrose before freezing gave preparations which upon thawing, centrifugation, and resuspension in 0.01 M KCl had the same activity as non-frozen preparations. Such preparations also exhibited a comparable CO_2 effect on the Hill reaction rate.

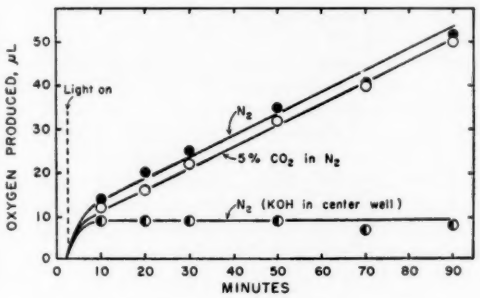
Quinone was purified by sublimation before each experiment.

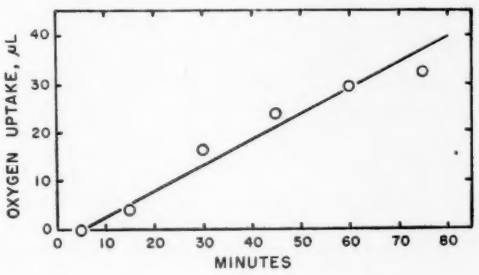
Chlorophyll concentration was determined spectrophotometrically by the method of Arnon (1).

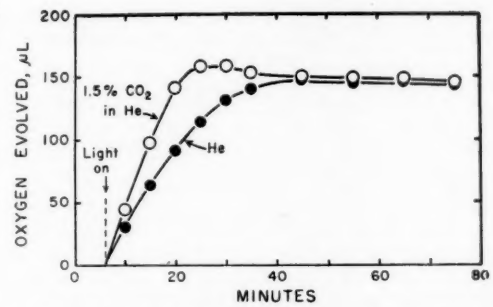
Manometric experiments were performed in the conventional manner. Vessels were shaken at 120 oscillations per minute at an excursion of 4.7 cm in a water bath at 20° C.

In manometric experiments light was supplied by a bank of six 300 photoflood bulbs 22 cm from the bottom of the Warburg vessels. These bulbs were run on 90 volts. In mass spectrometer experiments the light source was a 1,000 w condensed filament Mazda flood light with the image of the filament projected onto the plane of the vessel. The applied voltages are indicated in the data presented.

Experiments with a mass spectrometer were performed using a Nier-Consolidated instrument (Model 21–201) which had been adapted for continuous monitoring of the gas phase of the reaction vessel. This was a rectangular Warburg-type vessel of 15.8 cc total volume when attached to the joint which was in turn connected to the mass spectrometer inlet leak.







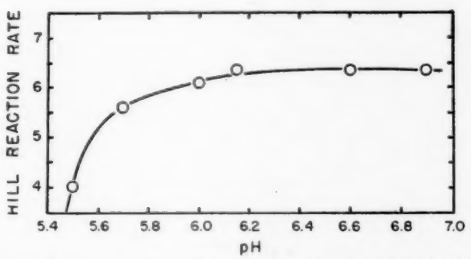


FIG. 1 (*upper left*). Time course of pressure changes in manometer vessels with and without CO_2 and with KOH in center well. Spinach chloroplast preparation and experimental conditions as specified by Boyle (2).

FIG. 2 (*upper right*). Time course of oxygen uptake in aerobic control vessel. Quinone in solution in main compartment; KOH in center well; chloroplast material omitted.

FIG. 3 (*lower left*). Oxygen production by Hill reaction. Kohlrabi chloroplast preparation, 2.5 mg chlorophyll per vessel. Quinone, 2.0 mg. Suspending medium, 0.01 M KCl. Liquid volume, 2.2 ml. Gas phase, helium ± 1.5 % CO_2.

FIG. 4 (*lower right*). Influence of pH on rate of Hill reaction. Kohlrabi chloroplasts suspended in phosphate buffer, 0.05 M.

The design of the joint and leak assembly and the method of shaking the vessel in a constant temperature bath did not differ in any essential particular from what was illustrated previously (8). The use of the mass spectrometer as adapted for the required measurements has been described (4). In those experiments in which tracers were used, isotopically enriched O_2 or CO_2 was introduced initially into the gas phase of the experimental flask; continuous monitoring of the gas phase with the mass spectrometer was carried on thereafter.

Brown and Weis (5) described the computations required to convert data obtained in the form of mass peak heights on the recorder chart to absolute measurements of gas evolution or uptake rates. This computation procedure includes the determination of isotope ratios of gases in solution from data obtained from gas phase measurements and takes into account the effects of diffusion lag of O_2 across the gas-liquid interface.

Argon was added to the gas phase in all mass spectrometer experiments and was monitored continually to facilitate correcting for machine drift and any other pressure changes due to non-biological factors.

Results

Using the triturated spinach leaf preparations described by Boyle, it was not possible to obtain an effect on the rate of the Hill reaction attributable to the presence or absence of CO_2. The rate was the same in 5 % CO_2 in N_2 as in 100 % N_2 (fig 1). When KOH was added to the manometer vessel center wells however, no pressure change occurred. This fact was found not to be due to the absence of CO_2 but rather to the reabsorbtion of all the O_2 evolved by the Hill reaction in the center wells. It is known that quinone will distill from the main compartment of a reaction vessel into the KOH solution. When this was measured quantitatively it was found that in an hour about 34 μg of quinone (1.7 % of total) was lost from the main compartment. Furthermore it was observed that, when quinone was absorbed on KOH-soaked filter paper in the center well under aerobic conditions, O_2 uptake occurred. The rate of uptake was found to be 29 μl O_2 per hour under conditions which simulated Boyle's (see fig 2). This rate was approximately equivalent to the rate of the Hill reaction as reported by Boyle and confirmed in the present study. Boyle's method of preparing chloroplast suspensions yielded preparations of low activity. He also used a remarkably high quinone concentration. Accordingly manometer vessels containing quinone in the main compartment, KOH in the center well, and air as the gas phase can be expected to show a rate of O_2 uptake sufficient to reabsorb all the oxygen produced by a Boyle-type Hill reaction. As shown in figure 1, it was possible to reproduce both qualitatively and quantitatively the results reported by Boyle. Nevertheless such results were spurious for the reason indicated.

Figure 3 shows the results of a manometric experiment with a kohlrabi chloroplast preparation designed to duplicate the conditions described by Warburg and Krippahl. These conditions were: 3 ml liquid volume, 0.1 % KCl, 2.1 mg quinone, and argon ± 1.4 % CO_2. Warburg and Krippahl provided no indication of the amount of chloroplasts added, the pH of the medium, or the use of a buffer. The contents of our vessels are described in the legend of figure 3. The light was turned on as soon as the

Table I

Carbon Dioxide Effect on Rate of Hill Reaction Determined by Warburg Manometry*

Experiment no.	% CO_2 in He	Chlorophyll mg/l	Rate**	%*** Diff.	% of Theoretical yield	%*** Diff.	Final pH of suspension
1a	0.001	560	5.75	+45.2	79.5	5.0	6.20
1b	5.0	560	8.35		83.5		5.80
2a	0.001	680	2.57	+83.5	82.5	3.0	6.00
2b	1.5	680	4.71		85.0		6.00
3a	0.001	432	7.05	+51.8	82.0	4.3	6.15
3b	1.5	432	10.7		85.5		6.00
4a	0.001	1116	2.64	+55.2	74.0	7.2	6.05
4b	1.5	1116	4.10		79.3		5.95
4c	0.001	1116	2.18	+47.9	78.0	8.3	6.00
4d	1.5	1116	3.23		84.5		5.90

* Liquid volume, 2.2 ml. Shaking rate, 120 oscillations/min. Excursion of vessel, 4.7 cm. Expected yield on the 2 mg of quinone per flask, 198 μl O_2. Temperature, 20° C. Data are averages for three vessels.

** μl O_2 minute^{-1} mg chlorophyll^{-1}.

*** Percent differences calculated as:

$$\frac{\text{avg. value for 3 vessels with } CO_2}{\text{avg. value for 3 vessels without } CO_2} \times 100$$

vessels had equilibrated and quinone had been tipped in from the side arm [3]. In this particular experiment the Hill reaction rate was 55 % higher in the presence of CO_2 than in its absence. The total oxygen yield was 7 % greater in the presence of CO_2. This latter difference was small but significant. (The average deviation of manometric results was less than 5 %.) The data illustrated in figure 3 are representative of a series of tests using different chloroplast preparations. Table I summarizes the results of five such experiments. The data are the averages of three vessels with CO_2 and of three without. The CO_2 content of the helium used to flush the vessels was measured with the mass spectrometer and shown to contain 0.001 % CO_2.

Because it had been found that a CO_2 effect on the Hill reaction could not be demonstrated consistently in the presence of phosphate, the experiments of table I were run without added buffer. Since changes in pH could lead to CO_2 retention or evolution thus influencing the pressure change, the pH was measured both before and after each manometric run. Chloroplast suspensions of course are buffered to some extent endogenously. Measured pH changes varied from zero to 0.4 units. At the CO_2 partial pressures which prevailed, such small changes in pH were quite insufficient to account for the differences observed manometrically between vessels which were provided with CO_2 and those which were not.

Furthermore the pH sensitivity of the Hill reaction itself also was not adequate to account for the difference in observed rates due to the presence of CO_2. A pH curve for the Hill reaction by kohlrabi chloroplasts is shown in figure 4. Flushing the vessels with gas containing CO_2 could be expected to cause some small decrease in pH of the suspension. This amounted to about 0.1 pH unit. At a pH of 6.0 to 6.2, according to the relation shown in figure 4, a change of 0.1 unit should have resulted in a barely detectable decrement in the Hill reaction rate rather than in an increase. Thus the CO_2 effect could not be attributed to the small pH changes which occurred in the poorly buffered suspensions.

[3] While this manuscript was in preparation a preliminary communication by Stern and Vennesland appeared. (J. Biol. Chem. 235: PC51–53. 1960.) These workers noted that the photochemical reduction of ferricyanide with catalytic amounts of dichlorophenolindophenol by spinach or kohlrabi chloroplasts could be made dependent on the presence of CO_2. The Stern and Vennesland effect seems to be different from the CO_2 effect of Warburg and Krippahl since Stern and Vennesland found a dark incubation time of several hours in the absence of CO_2 was required to elicit the effect. During the long incubation period Hill reaction activity declined with time more rapidly in the absence of CO_2 than in its presence. The addition of CO_2 quickly restored most of the Hill reaction activity. In our studies of kohlrabi chloroplasts using quinone as Hill oxidant rather than ferricyanide, prolonged incubation in the absence of CO_2 did not enhance the CO_2 effect.

The yield of O_2 was slightly but consistently larger when CO_2 was present than when absent (table I). Measurements of yield were taken as the highest point of the oxygen evolution time course.

The stimulatory effect of CO_2 on the Hill reaction also was observed using mass spectrometry in the place of manometry. Two sets of results are presented in table II. Aliquots of the same chloroplast preparation were used in all experiments within a set. Since these observations could not be replicated simultaneously with the apparatus presently available, it was necessary to perform successive experiments on comparable chloroplast samples. This necessitated preservation of Hill reaction activity by holding aliquots of the preparations in the frozen state until just before use. Within each set of experiments shown in table II the stimulatory effect of CO_2 was apparent. Because of differences in activities of chloroplast suspensions prepared at separate times from different sets of leaves as well as differences in

TABLE II

CARBON DIOXIDE EFFECT ON RATE OF HILL REACTION DETERMINED BY MASS SPECTROMETER*

EXPERIMENT NO.	% CO_2 IN He	RATE**	% OF THEORETICAL YIELD
		Set A	
5a	0.001	6.90	94.0
5b	0.003	6.05	74.0
5c	0.002	9.05	101.
5d	0.003	7.40	97.7
		Avg. 7.35	
5e	0.38	8.65	96.0
5f	0.14	8.65	95.5
5g	0.27	8.60	97.5
5h	0.72	8.95	101.
		Avg. 8.71	
		Set B	
6a	0.0005	3.03	85.1
6b	0.001	2.93	84.3
6c	0.001	2.98	81.4
		Avg. 2.75	
6d	0.58	4.25	84.5
6e	0.82	4.47	85.6
6f	0.61	4.32	85.5
		Avg. 4.35	

* Conditions:	*Set A*	*Set B*
Temperature	17° C	15° C
Chlorophyll conc.	344 mg/l	772 mg/l
Voltage supplied to light source	80 volts	110 volts

** μl O_2 minute^{-1} mg chlorophyll^{-1}

incident light intensity and chloroplast density, the rates of oxygen evolution in sets A and B are different. These data demonstrate convincingly that O_2 was indeed the only gas involved since the analytical method readily distinguished it from CO_2 and other gases.

Mass spectrometer experiments were carried out under aerobic and anaerobic conditions with and without CO_2. In all experiments the level of CO_2 initially present in the vessel remained unchanged. For those experiments in which tracer CO_2 was present the production and consumption rates were calculated independently from the tracer data. In all cases neither evolution nor uptake rates differed significantly from zero (table III).

It is difficult to present a single measure of reliability, but 10 % may be considered the limit of error of values computed from mass spectrometer data such as those presented in table III.

After the quinone had been reduced in the Hill reaction a simultaneous production and consumption of oxygen was observed. This was evidence for a Mehler reaction which would be expected under these conditions.

Some of the experiments in table III demonstrate an uptake of oxygen during the course of quinone reduction. Since these results fall within the range of error of the mass spectrometer method, it can be concluded that this does not contribute to the stimulatory effect of carbon dioxide on the rate of the Hill reaction. Besides, an uptake can be noted in both the presence and absence of carbon dioxide.

Warburg and Krippahl interpreted the CO_2 effect they had observed by reviving the discredited hypothesis that O_2 arises from the splitting of CO_2 by light. According to them the C resulting from that hypothetical cleavage reacts with quinone and water to form hydroquinone and CO_2 which again can be split and recombined in cyclic fashion. To test the hypothesis that the catalytic effect of CO_2 was achieved by a mechanism which involved the cyclic uptake and production of this gas by the chloroplast preparations, experiments were performed using CO_2 labelled with carbon 13. When a mixture of $C^{13}O_2$ and $C^{12}O_2$ was present in the gas phase, no consumption or production of either isotopic form of CO_2 was observed (table III).

Even though no cyclic metabolism of CO_2 could be detected, it must be realized that this in itself does not rule out the possibility that the environmental CO_2 was passing through a metabolic pool of chloroplast fixed carbon. So long as the turnover of such a hypothetical pool involves the production of the same carbon atoms which are incorporated, such a cyclic process could escape detection provided that the pool size is too small for changes to be observed in the isotopic ratio of the labelled CO_2. From the studies carried out with tracer carbon, it was calculated that a hypothetical pool of metabolic carbon compound could amount to no more than ½ % of the chlorophyll present and still escape detection in such experiments. On the other hand, if the hypothetical pool should equilibrate by rapid exchange with environmental CO_2 then it follows that an even larger pool would not be directly detectable by tracer experiments such as were performed.

TABLE III

PRODUCTION & CONSUMPTION OF OXYGEN & CARBON DIOXIDE DURING HILL REACTION DETERMINED BY MASS SPECTROMETRY*

GAS PHASE	EXP. NO.	CHLORO-PHYLL CONC.	% O_2, GAS PHASE	% CO_2, GAS PHASE	O_2, NET RATE OF CHANGE	O_2 PRO-DUCTION RATE	O_2 CON-SUMPTION RATE	CO_2 NET RATE OF CHANGE	CO_2 PRO-DUCTION RATE	CO_2 CON-SUMPTION RATE
Helium	7a	954	...	...	1.55	...	...	0.0	...	...
	7b	954	...	...	2.85	...	...	0.0	...	...
Helium	8a	410	...	0.18	3.25	...	...	...	0.015	0.18**
CO_2***	9a	1289	...	1.11	3.25	...	...	...	0.015	0.0
	9b	1280	...	0.33	3.56	...	...	...	0.0	0.0
Helium	10a	258	0.27	...	...	7.25	0.62**	0.0	...	...
O_2†	10b	128	0.49	...	...	5.15	—0.63	0.0	...	...
Helium	8b	410	0.35	1.15	...	4.42	—0.05	...	0.04	—0.09
CO_2***	10c	128	0.53	0.73	...	3.48	—0.56	...	0.08**	0.0
O_2†										

* Temperature, 15° C. Voltage to light, 80. Rates are expressed as $\mu l\ O_2\ min^{-1}\ mg^{-1}$ chlorophyll. In all cases gas evolution is recorded as +, gas consumption as —.

** Whenever calculations yield positive values for gas uptake or negative values for production, these are examples of experimental error.

*** Mixture of mass 44 and 45.

† Mixture of mass 32 and 34.

Conclusions

Boyle's observations with triturated spinach leaves were confirmed, but for the reasons discussed, such experiments do not demonstrate a catalytic CO_2 effect on the rate of the Hill reaction.

It was possible to obtain the catalytic effect of CO_2 described by Warburg and Krippahl though our results were quantitatively not as large. This effect is specific for oxygen evolution. There was no demonstrable production or consumption of CO_2 under conditions for which a CO_2 effect was observed. This finding fails to support but does not disprove the hypothesis that the CO_2 effect involves a rapid turnover of carbon.

An augmentation of the Hill reaction could not be noticed in experiments where both 0.5 M sucrose and 0.05 M phosphate buffer were present. Where phosphate buffer was used alone the CO_2 effect was reduced to about half of that of the 0.01 M KCl control. For vessels containing 0.5 M sucrose a CO_2 effect was demonstrable though the results were variable as compared to the control.

Sugar beet (*Beta vulgaris* L.) chloroplasts prepared in the same manner as the kohlrabi chloroplasts did not show a significant stimulation of the Hill reaction by CO_2. This CO_2 effect, therefore, is patently not as general a phenomenon as is Hill reaction activity itself.

Summary

Experiments by Boyle purporting to show a CO_2 stimulation of the Hill reaction were repeated. While Boyle's results were confirmed they were considered not to be evidence of a CO_2 effect per se. When alkali *was not* present in vessel center wells, the same rate of Hill reaction occurred with or without CO_2. When alkali *was* present, some quinone distilled from the main compartment onto the alkali-soaked filter paper in the center wells. The resulting mixture consumed O_2 at a rate comparable with the rate of O_2 production in the Hill reaction.

A report by Warburg and Krippahl of a CO_2 stimulation of the Hill reaction similar to that of Boyle but using different materials and methods was confirmed. With manometry and experimental conditions as specified by Warburg and Krippahl 1.5 to 5 % CO_2 was found to enhance the rate of the Hill reaction by approximately 50 %. Using mass spectrometry it was established that the CO_2 effect was specifically on the rate of O_2 evolution, not on O_2 uptake; nor was a gas other than O_2 evolved. No metabolism of CO_2 was observed. Using tracer CO_2 it was not possible to demonstrate a cyclic evolution-uptake of CO_2 which had been postulated by other investigators.

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ASSIMILATION OF AMMONIA BY NITROGEN-STARVED CELLS OF CHLORELLA VULGARIS [1]

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Introduction

In an investigation of the utilization of inorganic nitrogen by Chlorella, Reisner et al (17) measured the changes in the quantities of uncombined amino acids which occurred after adding nitrate or ammonia to nitrogen-starved cells. Adding ammonia produced more rapid and more striking changes in amounts of amino acids than adding nitrate. In the shortest period of ammonia treatment (15 min) marked increases in glutamine and alanine and a small increase in serine were observed.

To learn more about the short-term assimilation of ammonia by Chlorella, the following investigations were carried out: A: The concentration of several uncombined amino acids in Chlorella was measured 2 to 15 minutes after adding ammonium chloride; B: The effects of inhibitors on the concentration of amino acids, after adding ammonia, were determined; C: Enzymes that might be involved in ammonia utilization were studied; D: The amount of N^{15} in non-protein alanine, glutamine, and glutamate was measured after administering $N^{15}H_4NO_3$ for 1 and 5 minute intervals; E: The concentration of pyruvate and α-ketoglutarate was determined before and after adding ammonia to Chlorella; F: The effect of pyruvate and ammonia plus pyruvate on the quantity of amino acids was examined.

Materials & Methods

Stock Culture & Inoculation: *Chlorella vulgaris* Beijerinck var. viridis (Chodat) was obtained from the University of Indiana algal collection and maintained as described previously (17).

Growth of Nitrogen-Starved Chlorella & Preparation of Concentrated Cell Suspensions: Chlorella was grown on a low-nitrogen medium (17) containing 1 % glucose. One liter of sterile nutrient solution in a 4-liter Erlenmeyer flask was inoculated aspectically and shaken in continuous light (50 ft-c) on a reciprocal shaker (30 cycles/min) at 21° C. Lack of nitrogen resulted in a cessation of growth in 5 days at a packed-cell volume (16) of about ten milliliters per liter. After 5-day-old cultures were centrifuged at 2,000 *g* for 15 minutes, the supernatant solution was carefully removed. The cells were resuspended in the supernatant solution (which had been autoclaved & cooled) to give a concentrated uniform suspension containing approximately 1.5 ml of cells per 10 ml. Each sample comprised 10 ml of suspension.

Experimental Procedures: Additions to Chlorella suspensions were made in two ways. In some experiments, an initial control sample was taken and the remaining suspension was poured immediately into a flask containing dry material. Further 10-ml aliquots of suspension were removed at various times after mixing. In other experiments, a concentrated solution of test compound was added to the suspension. Immediately after mixing, one 10-ml portion was removed as a zero-time sample and subsequent 10-ml samples were obtained in the same way. During the incubation period, cells were shaken in light at 21° C. Separate 0.15-ml aliquots were taken to determine packed-cell volume (16).

For amino acid determinations, 10-ml aliquots of cell suspension were stirred into 50 ml of 95 % ethanol at −78° C. After warming to room temperature, the mixture was centrifuged and the supernatant solution was poured off. The residue was re-extracted with five successive 50-ml batches of 80 % ethanol at room temperature. The combined supernatant solutions were evaporated to dryness in vacuo on a rotary evaporator at 30° C and the resulting residue was dissolved in water. The extracted amino acids were purified on ion exchange resins (20) and quantitatively assayed by paper chromatography (19).

In enzyme studies, Chlorella cells were separated from the nutrient solution by centrifugation. The residual cell paste was weighed and cooled. After the cells were ground with five times their weight of levigated alumina in a chilled mortar, the resultant mixture was extracted with three volumes of cold 0.2 M tris[3] buffer at pH 8.15. The suspension was

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[3] The following abbreviations are used in this paper: tris = tris(hydroxymethyl) aminomethane; ATP = adenosine triphosphate; DNP = 2,4 dinitrophenol; DOP = deoxypyridoxine; DPNH = reduced diphosphopyridine nucleotide.

centrifuged and the supernatant solution was dialyzed over-night at 6° C against 0.001 M glutathione. The protein content was estimated by the method of Lowry et al (12).

Glutamic dehydrogenase was assayed by the Bulen method (1). Tests for alanine dehydrogenase were made by a similar method (22).

Glutamic acid-alanine and glutamine-alanine transaminases were measured by the incubation of dialyzed extract with glutamic acid plus pyruvic acid or glutamine plus pyruvic acid at 37° C for 60 or 120 minutes. The glutamic acid or glutamine and alanine were determined by reaction with ninhydrin on paper (19) after separation by one-directional paper chromatography in phenol and water (8:3 v/v). Glutamic acid-serine transaminase assays were made in a similar manner using hydroxypyruvate prepared from pyruvic acid (5).

Glutamine synthetase was measured by the method of Elliot (6).

Tests for the formation of alanine from pyruvate and adenyl amidate (2) were made by the procedures of Katunuma (9).

In experiments with N^{15}, the uncombined amino acids were extracted from cells with ethanol as described above. The amino acids from an entire sample were purified and chromatographed on 12 chromatograms (19). The amino acids were measured on one chromatogram by the quantitative ninhydrin reaction (19). The other chromatograms were sprayed with a 0.05 % ninhydrin solution in methanol. After 10 minutes at room temperature the glutamic acid, alanine, and glutamine spots and blank areas of paper of corresponding size were cut out (the other amino acids were omitted). The weight of paper was determined in each case. The paper pieces were dipped in KOH-borate buffer (4) and aerated at 40° C for 10 minutes to remove ammonia. The paper pieces were then eluted with 50 % ethanol.

The eluate of the glutamine spots was hydrolyzed with 1 N HCl for 2 hours at 100° C. The hydrolysate was made alkaline and the ammonia from the amide group was distilled into HCl. The remaining glutamic acid was digested with sulfuric acid as were the eluates of glutamic acid and alanine spots. The total ammonia in the digests and distillate was determined on a separate aliquot with ninhydrin (4). From eluates of blank areas on the chromatograms treated in the same manner, the contaminating nitrogen in the paper was determined. The remainder of the digests were made alkaline and the ammonia was distilled into 0.1 N HCl. The NH_4Cl obtained from the digestion of amino groups and from the hydrolysis of amide groups was converted to nitrogen with NaOBr and the N^{15} content was measured in a mass spectrometer.

Keto acids were extracted and converted to their dinitrophenyl hydrazones and the hydrazones purified by the procedures of Isherwood and Niavis (8). Appropriate portions were chromatographed on paper using *n*-butyl alcohol and NH_4OH (15). The two pyruvic acid spots and α-ketoglutaric acid spots were eluted with 1 % Na_2CO_3 solution (10).

Results

It was found previously that adding ammonium chloride to nitrogen-deficient Chlorella (17) resulted in a large increase in the content of non-protein alanine after 15 minutes. To learn if the alanine increment was apparent in shorter times, ammonium chloride was given to Chlorella for 2, 5, 10, and 15 minute intervals. Four amino acids showed significant changes (table I). In the shortest time (2 min) the alanine content showed the greatest increase of any acid both on an absolute and on a percentage basis. The synthesis of alanine appeared to take place immediately after adding ammonium chloride.

Within 15 minutes, glutamic acid decreased and then returned to its initial level. The decrease was certainly a consequence of glutamine synthesis. The increase in glutamine more than offset the decrease in glutamic acid resulting in a net increase in the total of glutamic acid plus glutamine.

Since it might be expected that DNP would inhibit glutamine formation and that DOP would inhibit transamination (23), the effect of these inhibitors on the assimilation of ammonia was examined (table I).

Table I

Uncombined Amino Acids in Chlorella Before & After Adding Ammonium Chloride

Amino acid	μmoles/ml of cells				
	Min after ammonia addition*				
	0	*2*	*5*	*10*	*15*
Glutamic acid	1.22	1.24	1.14	1.17	1.27
Glutamine	0.23	0.40	0.75	1.73	2.04
Glutamic + glutamine	1.45	1.64	1.89	2.90	3.31
Alanine	0.69	1.20	1.83	2.66	2.98
Serine	0.51	0.55	0.56	0.69	0.77
After 30 min incubation with 10^{-4} M DNP					
Glutamic acid	1.00	...	0.92	...	1.16
Glutamine	0.13	...	0.84	...	0.92
Alanine	0.79	...	1.64	...	1.96
After 120 min incubation with 10^{-4} M DNP					
Glutamic acid	0.97	...	0.82	...	1.05
Glutamine	0.15	...	0.89	...	0.98
Alanine	0.80	...	1.53	...	1.92
After 30 min incubation with 3×10^{-3} M DOP					
Glutamic acid	1.40	...	1.07	...	1.21
Glutamine	0.16	...	1.50	...	2.42
Alanine	0.55	...	1.96	...	2.93
After 120 min incubation with 3×10^{-3} M DOP					
Glutamic acid	1.29	...	1.07	...	1.14
Glutamine	0.15	...	1.40	...	2.04
Alanine	0.42	...	2.09	...	3.08

* 60 μmoles of NH_4Cl given per ml of cells.

Although DNP did not prevent the increase of alanine and glutamine between 0 and 5 minutes, changes in these acids between 5 and 15 minutes were much reduced. In the presence of DOP, glutamine was higher at 5 minutes and the same at 15 minutes as the untreated cells. These results are difficult to interpret. DNP did not inhibit glutamine formation in 5 minutes but did between 5 and 15 minutes irrespective of its time of contact with cells. Although DOP did not inhibit alanine formation this does not prove that alanine is not formed by transamination.

TABLE II

FORMATION OF ALANINE FROM GLUTAMATE & PYRUVATE IN PRESENCE OF CELL-FREE EXTRACT FROM CHLORELLA

REACTION MIXTURE	μmoles ALANINE FORMED/mg PROTEIN/HR
Complete*	0.37
Minus pyruvic acid	0.0
Plus boiled extract	0.0

* The complete reaction mixture contained 6.0 μmoles Na-pyruvate, 6.7 μmoles Na glutamate, 100 μmoles tris, and ca. 1 mg protein from the extract in a total volume of 2 ml. The mixtures were incubated for 1 hour at 37° C on a Dubnoff shaker.

Enzymes which might be responsible for the changes in amino acid content following ammonia addition, were assayed. Chlorella extracts have glutamine dehydrogenase (0.21 μmoles DPNH oxidized min/mg protein), glutamic-alanine transhydrogenase (table II) (cf. 15) and glutamine synthetase. No evidence was obtained for alanine dehydrogenase, glutamic-serine transaminase, glutamine-alanine transaminase, and animation of pyruvate with adenyl amidate (9).

If alanine were formed by direct amination of pyruvate, adding labeled ammonia (N^{15}) to Chlorella should result in a high N^{15} concentration in alanine. $N^{15}H_4NO_3$ was given to nitrogen-starved Chlorella cells for 1 and 5 minute intervals. Table III gives

TABLE III

CONCENTRATION & N^{15} CONTENT OF UNCOMBINED AMINO ACIDS OF CHLORELLA AFTER ADDING $N^{15}H_4NO_3$

AMINO ACID	MIN AFTER ADDING 0	1	5	$N^{15}H_4NO_3$* 1	5
	μmoles of Amino acid/ml of cells			*atom % excess of* N^{15}	
Glutamic acid	0.84	0.63	0.48	15.8	33.8
Alanine	0.53	0.89	2.18	6.5	20.7
Glutamine	0.09	0.32	0.59		
Amino group				16.2	44.4
Amide group				36.8	59.8

* 60 atom % of excess of $N^{15}H_4NO_3$ was used.

TABLE IV

PYRUVIC ACID & α-KETOGLUTARIC ACID CONTENT OF CHLORELLA BEFORE & AFTER ADDING AMMONIUM CHLORIDE

TIME AFTER ADDING NH_4Cl*	μmoles/ml CELLS	
	PYRUVIC ACID	α-KETOGLUTARIC ACID
0	0.35	0.16
5 min	0.51	0.17
15 min	0.51	0.23

* 60 μmoles per ml of cells.

the atom per cent excess of N^{15} in uncombined alanine, glutamine, and glutamic acid as well as the quantities of these acids. The notable point is that alanine has a much lower N^{15} content than glutamic acid. This result suggests that alanine is formed by transamination from glutamic acid.

If alanine is formed by transamination its rapid rise after adding ammonia could result from an increase in pyruvate or a decrease in α-ketoglutarate (since glutamic acid does not change much). The keto acid content of Chlorella before and after adding ammonia was determined (table IV). Pyruvic acid level increased about 50 % and α-ketoglutaric acid to a lesser extent.

TABLE V

EFFECT OF PYRUVIC ACID & AMMONIA ON CONTENT OF UNCOMBINED ALANINE & GLUTAMIC ACID IN CHLORELLA

TIME IN MIN AFTER ADDING INDICATED COMPOUNDS		CONTENT OF AMINO ACIDS	
PYRUVIC ACID*	NH_4Cl*	ALANINE	GLUTAMIC ACID
		μmoles/ml of cells	
0	0	0.73	0.86
30	0	0.88	0.81
32	2	1.24	0.67
35	5	1.94	0.59
45	15	2.23	1.26
0	2	0.93	0.73
0	5	1.39	0.64
0	15	2.98	0.97

* 60 μmoles/ml of cells.

Since pyruvic acid increased on the addition of ammonia, the effect of adding pyruvic acid either without or with ammonia on the alanine level was determined. Data on alanine and glutamic acid only are included since glutamic acid is the most likely donor in transamination to alanine (table V). Although the addition of pyruvic acid increased the alanine content and decreased the glutamic acid content

somewhat in 30 minutes, it is clear that the prior incubation with pyruvic acid does not change the extent of the subsequent increase in alanine content after the addition of ammonia.

Discussion

The central role of glutamic acid in nitrogen metabolism of plants has been emphasized repeatedly (3, 11, 24, 25). After the feeding of N^{15}-labeled ammonia to plants, the relatively high N^{15} content of glutamic acid has been noted (13, 21, 25). More recent work of Sims with yeast (7) and Cocking with barley (7) has also shown that after giving N^{15} ammonia for relatively short periods (16 min & 2 hrs, resp.) glutamic acid has the highest N^{15} content. Yemm and Folkes (24) state that in yeast, over 90 % of ammonia may be assimilated by the glutamic acid pathway. This conclusion is particularly interesting since Roine (18) had observed a rapid rise in alanine after giving ammonia to nitrogen-deficient yeast.

In the Chlorella experiments reported here it was found that the N^{15} content of glutamic acid was considerably higher than the N^{15} content of alanine after the administration of labeled ammonia. Assuming a negligible metabolism of alanine in 1 minute of ammonia feeding, it can be calculated that the 6.5 atom % excess of N^{15} in alanine must have derived from a compound with about 16.0 % N^{15}. This suggests that the alanine nitrogen derives from glutamic acid (since transamination from glutamine to pyruvate could not be demonstrated). This conclusion agrees with the inability to demonstrate any mechanism for the direct formation of alanine from ammonia. The N^{15} data show that even if there were direct pathways of alanine formation they are not significant under these experimental conditions and could not account for the alanine increase after ammonia feeding. Furthermore, the presence of an active glutamic acid dehydrogenase and glutamic acid-alanine transaminase in Chlorella shows that these enzymes could provide the pathway for alanine formation.

Summary

The mechanism of the alanine increase in nitrogen-starved Chlorella following the addition of ammonium chloride was investigated. Within 2 minutes alanine showed a large rise. Pyruvic acid increased 50 % in 5 minutes and α-ketoglutaric acid increased to a lesser extent in 15 minutes. Adding pyruvic acid to a Chlorella suspension resulted in a small increase in alanine and a small decrease in glutamic acid. Dinitrophenol and deoxypyridoxine had little effect on glutamic acid and alanine levels.

After feeding $N^{15}H_4NO_3$ to Chlorella for 1 and 5 minutes the amide group of glutamine showed the greatest N^{15} enrichment. The amino group of glutamine and glutamic acid had the next highest N^{15} content while that of alanine was lower. These results are consistent with incorporation of ammonia into organic combination catalyzed by glutamic dehydrogenase and glutamine synthetase. These enzymes and glutamic acid-alanine transaminase are present in Chlorella. Glutamine-alanine transaminase and enzymes for direct amination of pyruvic acid were not found. An ammonia-induced increase in pyruvic acid may be partially responsible for the increase of alanine, which occurs directly after administration of ammonia to the low-nitrogen cells.

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EXTRACTABILITY OF DNA & ITS DETERMINATION IN TISSUES OF HIGHER PLANTS [1, 2]

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RESEARCH BRANCH, CANADIAN DEPARTMENT OF AGRICULTURE

INTRODUCTION

A number of methods have been described for determining deoxyribonucleic acid (DNA) in higher plants. Three of these are commonly used: cold and hot perchloric acid extraction (Ogur & Rosen, 5), hot salt extraction (Martin & Morton, 4), and alkali extraction followed by acid hydrolysis (Schmidt & Thannhauser, 6). In some procedures, DNA is first separated from ribonucleic acid (RNA), in others not; in both cases, the analysis of choice has been the measurement of deoxyribose content by means of the diphenylamine reagent (1). Ultra-violet absorption or phosphorous content have been used instead of the specific sugar reagent, but in general, these remain unreliable indicators of DNA concentration.

Interests in some developmental problems such as cell-division, root differentiation, and tumor formation had led us to examine the relative merits of the above procedures with a view to selecting a fully reliable one. This has only been partly accomplished; differences between tissues prohibit a single formulation.

METHODS

The procedures used in determining the DNA content of plant tissues may be divided into three parts: I: Preparation of finely disintegrated fat-free powders; II: Extraction of DNA by means of a suitable solvent; III: Determination of DNA concentration in the extract by an appropriate analytical technique.

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I. PREPARATION OF FAT-FREE POWDERS. *A. Soft tissues (e.g. root tips, anthers).* These, in quantities up to 10 g fresh weight, were disintegrated by means of a conical glass homogenizer (Kontes Glass Co., Vineland, N. J.) in cold methanol containing 0.05 M formic acid. Treatment of tissues with acidified methanol facilitates extraction of pigments, and possibly other substances. For some tissues, such as root tips, methanol alone appeared to be satisfactory. Larger quantities of tissue were first treated in a high-speed blender and then further disintegrated in a glass homogenizer. The suspension thus obtained was centrifuged at 0 to 2° C and the clear supernatant fluid was discarded. The residue was redispersed in methanol-formic acid and again centrifuged. This step was repeated until the supernatant fluid appeared colorless. The tissue was then suspended in a mixture of ethanol: ethyl ether (2:1, by volume), and allowed to stand for 30 minutes at 50° C. The suspension was then centrifuged and the supernatant fluid discarded. The residue was washed twice with ether and dried in vacuo.

B. Fibrous tissues (e.g. stems). These were cut into lengths of 1 or 2 cm and mixed with dry ice. The mixture was put in a nylon bag and pounded with a mallet to yield a coarse powder, which was then passed through a Wiley Mill. Care was taken to have enough dry ice present to prevent thawing of the tissue. The mixture of disintegrated tissue and dry ice was collected in methanol-formic acid and the suspension allowed to warm to a temperature of 0 to 2° C. Subsequent steps were the same as in A.

II. DNA EXTRACTION. The Ogur-Rosen (1) and Schmidt-Thannhauser (3) procedures for extraction of fat-free tissue powders were followed without significant modification. Extraction with hot salt solution was done in the following way: An appropriate weight of tissue powder was dispersed in a pyrex ignition tube with a minimal volume of 10 % NaCl (W/V) containing 0.05 M tris (hydroxyamino) methylmethane (tris) buffer at a pH of 7.0. The tube was placed in a boiling water-bath and after about 1 minute of warming, it was tightly stoppered. The slight positive pressure thus maintained during extraction prevented the tissue powder from floating. After 30 minutes the tube was removed from the bath, cooled, and centrifuged, the supernatant fluid being retained. In some cases (see Results) one or two additional extractions were necessary. The extract was made 0.5 N with respect to perchloric acid and heated for 20 minutes at 70° C. In cases where a precipitate remained after heating, the extract was clarified by centrifugation.

III. DNA ANALYSIS. The Dische reaction (2) as modified by Burton (1) was followed. Redistillation of diphenylamine, as recommended by Burton, was found to be unnecessary if the reagent was prepared as follows: The entire contents of a newly-opened bottle of diphenylamine (Reagent Grade, Fisher Scientific) were dissolved in glacial acetic acid at a final concentration of 100 g/l of solution, and stored in a dark bottle in a refrigerator. The solution was stable for at least six months. A stock reagent was prepared by diluting 60 ml of the stored solution to 400 ml with glacial acetic acid and adding 6 ml of concentrated H_2SO_4. The stock reagent solution was kept at room temperature. Diphenylamine purchased from some commercial sources yielded colored solutions which were discarded. Just before use, 0.1 ml of an acetaldehyde solution (16 mg/ml H_2O) was added to each 20 ml of stock reagent. Two volumes of reagent were added to one volume of appropriately diluted extract and the mixture incubated for 18 hours at 25 to 30° C. The optical densities of the color formed on overnight incubation were determined at 600 and 540 mμ, and the difference between the values used to compute the DNA concentration. A standard solution was run with each test. Polymerized DNA from commercial sources is commonly used as a reference standard but we found deoxyadenosine to be more reliable for this purpose, and also easier to use. A solution of deoxyadenosine in 50 % ethanol was made up to a final concentration of 0.5 μmoles/ml and 0.1 ml of this used in each set of analyses. One μmole of deoxyadenosine is equivalent to 2 μmoles DNA-P.

In assaying saline extracts for DNA content by the diphenylamine reaction, it was found necessary to limit the chloride ion concentration in the final mixture to 0.7 %. Above that value there was a progressive decrease in color intensity with increasing chloride ion concentration. Those saline extracts having a low DNA concentration (as was the case for older regions of stems) were therefore first lyophilized to yield a dry residue, which was washed two to three times with a cold 0.72 M trichloracetic acid to remove the NaCl. The washed residue was suspended in a suitable volume of 0.5 N perchloric acid, heated for 20 minutes at 70° C, then cooled and centrifuged, the supernatant fluid being retained for analysis. Generally, one such extraction with hot acid was sufficient. In some cases, however, two extractions were necessary to bring all of the DNA into solution.

The specificity of the diphenylamine reaction was checked by the microbiological assay method of Hoff-Jorgensen (3). The assay was performed either directly on small amounts of defatted tissue powder or on portions of the saline extracts. In essence, the procedure consisted of treating the powder or extract with deoxyribonuclease to degrade the DNA to acid-soluble polydeoxyribonucleotides and then hydrolyzing the latter to deoxyribosides by means of alkaline phosphatase. Although this assay of DNA is less precise than the diphenylamine reaction, its absolute specificity makes it possible to determine if an appreciable proportion of the color produced in the presence of diphenylamine is due to constituents other than deoxyribose.

TABLE I

DNA CONTENT OF CORN ROOT SEGMENTS

SEGMENT	DIRECT ASSAY	SALT EXTRACTION		PERCHLORIC ACID EXTR.
	MICROBIOLOGICAL	DIPHENYLAMINE	MICROBIOLOGICAL	DIPHENYLAMINE
1	84	80	82	86
2	55	56		53
3	33	28	30	32
4	29	23		26
5	25	22	22	22

Values are expressed as μg DNA–P/100 mg of defatted powder. The latter was extracted three times with either 10 % NaCl or 0.5 N perchloric acid. Other procedures are described in text. Each of the successive segments was cut to 3 mm length.

RESULTS

For meristematic tissues, and, in some instances, those proximal to them, the Ogur-Rosen procedure is satisfactory, and possibly, the one of choice. This is shown in an analysis of corn root segments up to 15 mm from the tip. 0.5 N perchloric acid extracts as much or a little more DNA than 10 % NaCl (table I). Indeed, in the alga Chlorella, salt is a poor extractant (8).

Mature tissues do not respond to DNA extraction in the same way as meristematic ones; neither do they behave uniformly. Leaves of wheat and tobacco and stems of *Vicia faba* are more effectively extracted with salt solutions. The DNA of wheat roots is extracted equally well with salt or perchloric acid, but only after five hot perchloric acid extractions, each for 20 minutes; by contrast, one salt extraction removes more than 90 % of the DNA. The behavior of wheat leaves with respect to DNA extractability is illustrated in figure 1. The curves are typical for those tissues in which salt solution is the more efficient extraction medium. For reasons we have not explored, one extraction with hot perchloric acid followed by four washes yields only a little more DNA than the first extraction alone. 1.0 N perchloric is no more effective than 0.5 N; nor does prolongation of individual extractions substantially affect the result. Similar results were obtained with tobacco leaves. Thus, one extraction with hot 10 % NaCl yields a value of 32 μg DNA–P per 100 g powder, five hot perchloric extractions yield a value of 25, and direct microbiological assay of the powder, 31. On the basis of these experiments it would appear that mature plant tissues are best extracted with hot salt solutions but no firm predictions can be made about untested species.

The Schmidt-Thannhauser procedure was then tested by incubating powders of the different plant tissues in 0.1 N NaOH at 37° C for 18 hours, and precipitating the extracted material with 0.72 M trichloroacetic acid. Both residue and the acidic supernatant fluid were tested with the diphenylamine reagent. Here too results vary when comparisons are made between alkaline and saline extractions. Wheat embryos and tobacco leaves yield nearly equivalent values by either method; wheat leaves and roots do not (table II).

The behavior of wheat leaves is somewhat perplexing since direct microbiological analysis of defatted powders yields significantly lower values than diphenylamine analysis of saline extracts. The possibility that a second tissue component contributes to the diphenylamine reaction cannot be excluded, although it is also possible that DNAase does not completely hydrolyze the DNA in the powder. Apart from this disparity in results the data are consistent in showing that where alkaline extraction of DNA is less effective than salt, it is also less effective than enzymatic degradation and microbiological assay.

There is a facet to the phenomenon of DNA extractability from plant tissues which would appear to have a significance beyond that pertaining to quanti-

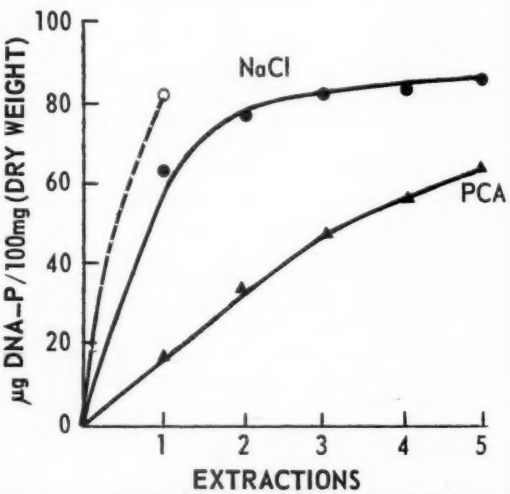

FIG. 1. Sequential extraction by salt or perchloric acid (PCA) of defatted powder prepared from wheat leaves. The dotted line represents one extraction with salt. In this case, the DNA content was calculated from the concentration of DNA in the saline extract multiplied by the total volume of the suspension prior to centrifugation.

TABLE II

ALKALINE EXTRACTION OF DNA

PLANT	DIRECT ASSAY MICROBIOLOGICAL	SALT EXTRACTION	NaOH EXTRACTION		
			TOTAL	PRECIPITATE	SUPERNATANT FLUID
Wheat embryos	...	137	121	121	...
Tobacco leaves[1]	31	34	29	27	2
Wheat leaves	72	87	60	56	4
Wheat roots	55	54	41	35	6

[1] In this case only the residue after NaOH extraction was further extracted with hot 10 % NaCl, and found to contain 2 μg DNA–P. Values are expressed as μg DNA–P/100 mg. defatted powder. The diphenylamine reaction was used in these determinations. Under "NaOH extraction", values are given for the acid-precipitable portion (precipitate) and for the acid-soluble one (Supernatant Fluid).

tative estimations. This is the variable solubility of DNA in 0.25 M NaCl. Initially it had been observed that in the development of lily anthers, there exists a cycle of rise and fall in such solubility (8). Commonly, much RNA but little DNA is extracted in dilute salt solutions. In a study of the broad bean (*Vicia faba*), however, it was found that depending upon conditions of growth the proportion of DNA which could be thus extracted increased with the age of the stem internode. The kind of pattern found in plants grown in a greenhouse is indicated in table III. Less striking differences were observed in plants grown under constant temperature conditions. To test the possibility that the DNA measured by extraction with cold 0.25 M NaCl might have been due to enzymatic degradation, the yields of DNA from tissues homogenized in cold 0.25 M NaCl were compared under the following conditions: A: The suspension was centrifuged immediately after preparation. B: The suspension was allowed to stand for 2 hours in an ice-bath, then centrifuged. C: Homogenization and centrifugation were carried out at room temperature. Similar values were obtained under all three conditions.

We have not determined in any definitive way the nature of the relationship between age of internode and DNA extractability. In mature bean stem segments, the proportion of DNA extracted with 0.25 M NaCl varied between 30 to 80 %. Since the conditions of extraction do not appear to be the source of the variation, we presume some physiological factor to be operative.

TABLE III

DISTRIBUTION OF DNA EXTRACTED WITH 0.25 M NaCl IN INTERNODES OF BROAD BEAN STEM

INTERNODE	TOTAL DNA	EXTRACTABLE DNA	% EXTRACTABLE TOTAL
2	9.1	0.22	2.3
3	7.4	1.22	17
4	7.4	3.0	41

Internodes are numbered from tip of stem. Extractable DNA was obtained by use of 0.25 M NaCl buffered with 0.05 M tris(hydroxyamino)methlymethane (pH 7.2). In early experiments 0.05 M sodium citrate was also added but this was later omitted as no protective effect was observed. pH of the suspension was checked soon after preparation and the pH adjusted if too acid. Generally a Spinco preparative centrifuge run at 90,000 × *g* was used to obtain clear extracts. Values are expressed as μg DNA–P/internode. DNA was determined both chemically and microbiologically.

CONCLUSION & SUMMARY

With respect to the quantitative determination of DNA in tissue of higher plants, we have outlined certain procedures which appear to provide reliable values. We have indicated the limitations imposed upon any one method by the type of tissue studied. The differences between meristematic and mature tissues have been particularly emphasized.

We have further indicated that the ease with which DNA may be extracted by mild procedures is also related to maturation. It seems likely that such extractability may be dependent upon the protein moiety of the DNA-protein complex.

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DNA CONTENT OF BROAD BEAN (VICIA FABA) INTERNODES IN CONNECTION WITH TUMOR INDUCTION BY AGROBACTERIUM TUMEFACIENS [1, 2]

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INTRODUCTION

This paper is concerned with changes which occur in the deoxyribonucleic acid (DNA) content of *Vicia faba* during tumor induction by *Agrobacterium tumefaciens*. Thus far, the phenomenon has been described in several ways. Oft quoted is the work of Klein (2, 3), who claimed that unlike responses to wounding or inoculation with attenuated bacteria, tumor induction by a virulent bacterium evokes first a marked increase in DNA. His analyses were made by conventional chemical methods. Rasch, Swift, and Klein (5) analyzed infected tissues by microspectrophotometric methods and found no evidence for a sharp increase in DNA during the inductive phase. Robson, Budd, and Yost (6) compared tissue cultures of tumorous and non-tumorous tissue and concluded that tumor cells contain more DNA than normal ones and that the excess may be of non-chromosomal origin. The latter conclusion might be interpreted to mean that induction of tumor is tied to some novel pathway of DNA metabolism.

Simply put, the problem is reduced to ascertaining whether direct chemical analysis of plant tissues during the tumor induction phase is or is not at variance with histochemical measurements. At issue is the nature of the primary act of the tumor-inducing principle. If a marked increase in DNA such as noted by Klein (2, 3) precedes other observable events, then a primary alteration in DNA metabolism would appear likely; if the contrary is true, then subtler changes such as those discussed by Braun and Stonier (1) must be sought for.

MATERIALS & METHODS

Vicia faba (L.) (var. Broad Windsor) was used exclusively. *Agrobacterium tumefaciens* (Smith & Townsend) (strain 3) was the infective agent. Strain B-6 was tested but only small overgrowths were produced. Plants were inoculated with a suspension of strain 3 bacteria using a hypodermic syringe with a 24-gauge needle. Wounded controls were plants inoculated similarly but with sterile water. Punctures were made as closely as possible along one entire face of an internode and the needle pushed through the stem so that opposite faces were similarly wounded. Two adjacent internodes of each plant were thus infected. The three groups of plants selected for comparison (controls, wounded, & infected) were as similar as possible in terms of total size and length of corresponding internodes. Generally, 7 to 14 plants were used per group. At desired time intervals, internodes were cut, weighed, and frozen immediately. Subsequent treatment of tissues has been described in the preceding publication (6).

RESULTS

In pursuing our primary objective—a comparison of DNA changes between healthy, wounded, and infected plants—it became clear after four or five sets of experiments that absolute values of DNA contents, based on fresh weight or dry weight, could not be used for comparative purposes. The situation arises from two growth characteristics of the plant. First, the changes in DNA relative to increase in weight differ from one internode to another (fig 1). Older internodes change little; younger ones show a progressive decrease in DNA per unit weight. It is clear, therefore, that not only must corresponding internodes be used for comparison, but also that DNA content should be expressed on a per internode basis. This method of computation avoids complications which might arise from the effect of wounding or infection on the rate of increase in mass. Furthermore, comparisons between corresponding internodes are much affected by the overall size of a plant (fig 2). In the larger of the two plants used for illustration the difference in DNA content between internodes 2 and 3 is much less than that observed in the smaller plant. Much the same holds true for relative weights of adjacent internodes (fig 2). The desirability of comparing plants with similar over-all height is therefore patent. We also noted marked differences in DNA content of groups of corresponding nodes depending upon the day of harvest. Temperature was probably the critical factor, since its effect on growth rates could markedly affect DNA contents.

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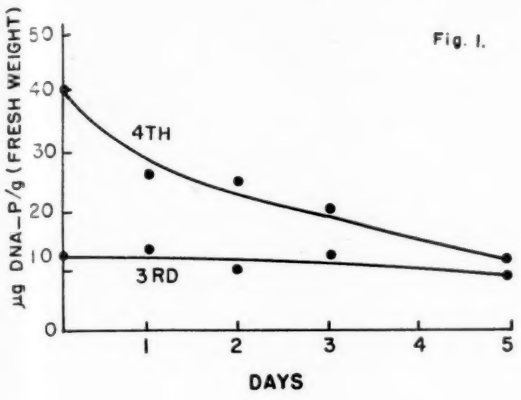

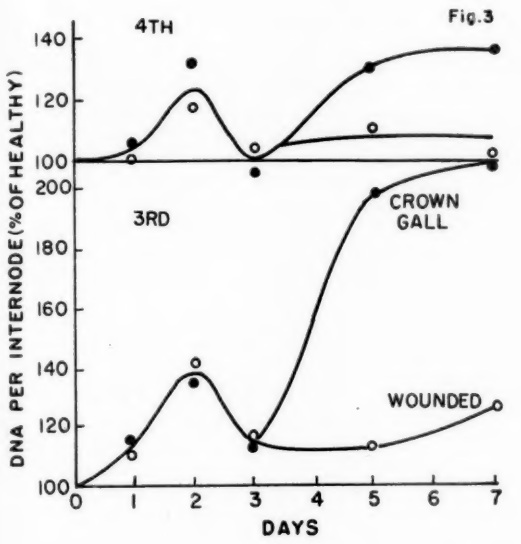

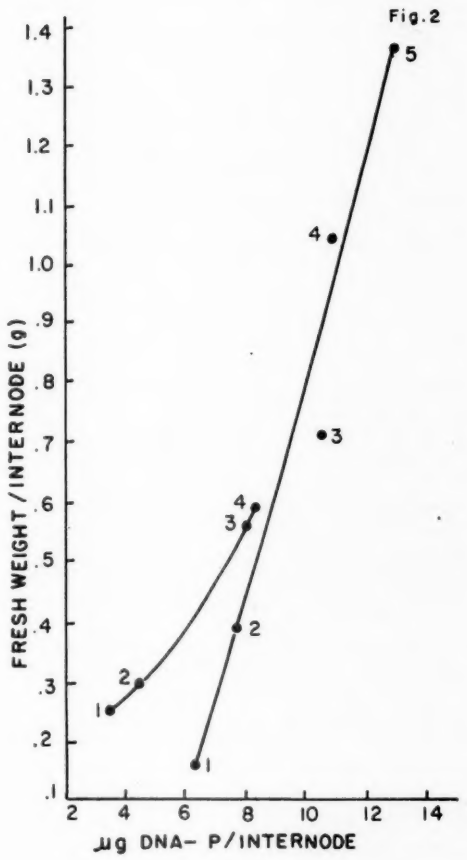

FIG. 1. Changes in DNA concentration during growth of internodes 3 and 4. In this figure internodes have been numbered from the base. The older internode (3) has undergone little growth in 5 days and consequently the DNA remains nearly constant. By contrast, cell enlargement has occurred in fourth internode but little mitosis and hence the fall in DNA concentration. Each point in the graph represents an analysis of a group of ten plants. All of the groups were matched as closely as possible for size.

FIG. 2. The DNA content of sequential internodes in two bean plants. The slopes of DNA content and weight of corresponding internodes differ markedly in smaller (*upper curve*) and larger plant. Internodes are numbered from apex in this figure only.

FIG. 3. Changes in DNA content of wounded and infected stems. Note the greater relative change in the older third internode. Each point represents an analysis of seven internodes. Conditions of experiment described in text.

To minimize the possible effects of variations, experiments were conducted in the following way: Three groups of plants were selected from a relatively large population by repetitive matching of sets of three in which both size of plant and size of internodes 3 and 4 were as close as possible. The plants were labelled and randomly placed for growth. Inoculations and woundings were completed within a day and harvests of each group were made at the same time (Plotted in fig 3). The DNA content of wounded and infected internodes is expressed as a percentage of the healthy ones. One conclusion which may be drawn from the figure is that wounded and infected plants behave similarly with respect to DNA during the first three days following stem puncture. Since the curves are, in effect, measurements of relative rates of DNA formation, it follows that during the induction period, no quantitative departure occurs in rates of DNA synthesis of infected plants. The effect of temperature on the time at which wounded and infected tissues depart from one another with respect to DNA content is shown in figure 4. These differences in time of DNA change parallel those observed by Braun (5) with respect to time of tumor induction.

The curves in figure 3 present a second interesting feature. In both infected and wounded plants there is a transient increase in relative *rate* of DNA formation between the 1st and 2nd days, the content thereafter returning to the level of the healthy ones. The time at which this increase in rate occurs varies with temperature but it is a property easily spotted when simultaneously comparing differently treated groups of plants. It is also the kind of change which Klein presumed to be characteristic of infected plants alone. We were therefore interested in determining the cause of this rate increase.

The cause appears to lie in the effect which wounding produces on internode growth. In figure 5 the increase in lengths of internodes 3 and 4 is compared in normal, wounded, and infected plants. The pattern of behavior is clear: Wounding, whether in the presence of sterile water or of *Agrobacterium tumefaciens*,

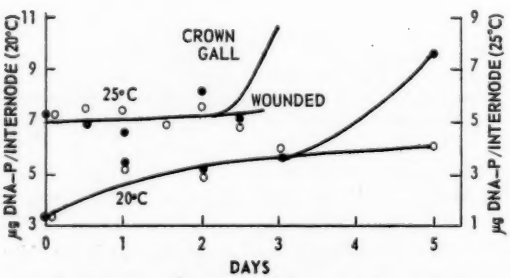

FIG. 4. Effect of temperature on changes in DNA concentration of internodes 3 and 4 in wounded and infected plants. Each point is based on the analysis of 7 to 10 internodes. A growth chamber was used to obtain temperature differences.

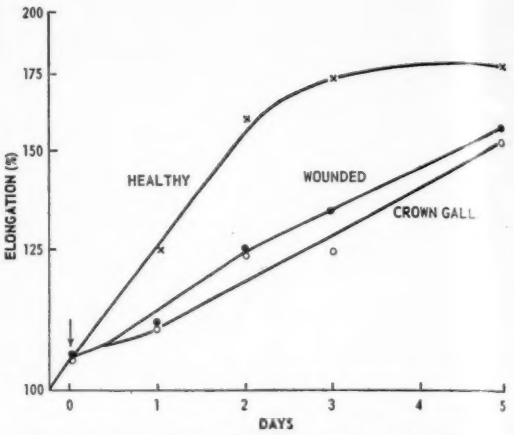

FIG. 5. Changes in rate of elongation of internodes 3 and 4 in normal, wounded, and infected plants. In each group of seven plants, the combined lengths were used for calculation because of the observation that either of two adjacent internodes might be the more severely affected. The arrow indicates time of wounding.

transiently inhibits internode growth. While we do not know the biochemical counterpart of this inhibition, it is apparent that DNA synthesis is not only unaffected but is temporarily stimulated relative to the normal plant. Thus, while one consequence of wounding is a heightened DNA synthesis, such synthesis is not a distinctive response to the action of the tumor-inducing principle. We do not know if successful tumor induction is dependent upon such heightened DNA synthesis.

Finally, we considered the possibility of distinctive differences existing in DNA extractability between infected and non-infected plants. The variability in amount of DNA extractable with cold dilute salt under different conditions (4, 7), suggested to us that such variability might have its seat in the non-chromosomal DNA postulated by Robson et al (6). Many experiments were made and many curves drawn but none of these pointed convincingly and consistently to a distinctive difference between the three groups of plants. It may be that cultured tumors do exhibit such a difference but we have not analyzed these.

CONCLUSIONS

The question to which these experiments has been addressed is whether or not the genesis of crown-gall tumors has its origin in an alteration of the DNA synthesis mechanism that otherwise might limit cell proliferation in healthy plants. To the extent that the alteration is quantitative the answer appears to be negative. There is no doubt that a net consequence of tumor induction is heightened DNA synthesis, but in so far as DNA measurements serve as a criterion, such heightened synthesis is not the initial act in

tumor induction. Direct chemical measurements of DNA are thus in agreement, not in discord, with histochemical measurements (5), at least for the first 5 or 6 days following infection. DNA changes which do occur in a growing plant following infection with *Agrobacterium tumefaciens* are the result of puncturing and thus also occur in wounded tissues.

The absence of marked quantitative changes in DNA content during initial stages of tumorogenesis does not imply the absence of change in regulatory mechanisms governing DNA synthesis. Our studies, however, do not penetrate to this problem. We may only conclude that if a regulatory change does occur, it is not manifest as an early increase in the rate of DNA synthesis.

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RAPID BIOASSAY FOR KINETIN & KININS USING SENESCING LEAF TISSUE[1]

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The isolation and characterization of kinetin (6-furfurylaminopurine) was first reported in 1955 by Miller, Skoog, von Saltza, and Strong (4). Since then both kinetin and a number of its analogs have been shown to have remarkable effects on plant growth (1, 2, 5, 7). So far, however, no natural kinin has been isolated and identified. The search for natural kinins and their subsequent isolation has been hampered by lack of a fast and simple bioassay. To meet this need we have developed the following quantitative assay which is based upon the ability of kinetin to retard the degradation of chlorophyll in the cells of senescing leaf tissue (7). It is quantitative over the range from 0.05 to 5.0 μg of kinetin and may be used for detecting still larger amounts, although it is then no longer quantitative. The assay may be used either to test zones cut directly from paper chromatograms or for material in solution. Unlike tests for kinetin based on growth responses (3), the present method does not require the addition of auxin.

Material & Methods

Cocklebur plants (*Xanthium Pensylvanicum* Wall.) of the inbred strain used in the California Institute of Technology are grown from seed in a greenhouse (max temp 30° C, min 20° C; air desmogged with charcoal filters). Normal daylight is supplemented with incandescent light to give a 20 hour photoperiod. Under these conditions the mature leaves are thin and soft in texture and attain an area

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[3] Supported by a National Research Council of Canada Special Scholarship.

of about 150 cm². When the plants have produced ten leaves in all, the fifth fully-expanded leaf (counting back from the apex) is suitable for the bioassay. The leaves are harvested and stored with the petioles in water under conditions of low light intensity (50 ft-c) for 3 days in Plexiglas boxes at 20° C. During this period, metabolic processes leading to senescence are initiated and there is a fall in the total chlorophyll content of the leaf. The final selection of suitable leaves is made on the basis of a uniform pale green color of the blade.

Kinetin standards are prepared as follows: 0.5 ml of aqueous solutions of kinetin at concentrations ranging from 16 to 0.063 mg/l are applied uniformly to circles of Whatman no. 1 filter paper, 4.25 cm in diameter placed in the base of a 5 cm petri dish. Control circles receive 0.5 ml of distilled water. Paper for the chromatography of extracts must be thoroughly washed to avoid accumulation of toxic materials just behind the solvent front. Ethyl acetate, acetic acid, water, (10:5:2, V/V) (8) is very effective for this purpose. Uniform discs 12 mm in diameter are cut from the interveinal blade tissue of previously aged and selected cocklebur leaves. Some 60 discs may easily be obtained from a suitable leaf. Four discs are placed on each filter-paper circle or portion of chromatogram so that the abaxial surface of the leaf tissue is in contact with the liquid. (0.5 ml is a suitable volume of water to add to portions of chromatogram strip large enough to accommodate four discs.) Duplicate sets of four untreated discs may be extracted immediately in boiling 80 % ethanol and the extract retained for determining the original chlorophyll content of the discs. Each small dish is placed on two layers of damp filter paper inside a larger closed petri dish. The larger dishes are stacked in enamel trays lined with damp filter paper and covered with aluminum foil. They are then maintained in darkness for 48 hours at 24° C. By the end of this period most of the chlorophyll in the controls is degraded and the discs are yellow. Those in the higher kinetin treatments are still green. (If the leaves have been insufficiently aged prior to use the duration of the assay may have to be extended.)

Each group of discs is then dropped into a graduated centrifuge tube containing 5 to 6 ml of hot 80 % ethanol, and boiled gently on a water bath until the chlorophyll has been extracted. The tubes are cooled and the volume made up to 10 ml with 80 % ethanol. The optical density of each solution was measured against 80 % ethanol in a Beckman spectrophotometer, Model B at 665 mμ and 645 mμ, the absorption maxima in the red region for chlorophyll a and chlorophyll b, respectively.

Results

The optical densities of extracts of leaf discs which had been exposed to kinetin standards ranging from 0.031 to 25 μg are presented in figure 1. It is clear that since the chlorophyll a/chlorophyll b ratio remains approximately the same in the presence of kinetin the determination of the optical density of the extract at either the chlorophyll a maximum or the chlorophyll b maximum is suitable as a measure of the retention of total chlorophyll in the leaf tissue. However, since it is advantageous to cover a wide range of optical densities, measurement at the wave length of the maximum absorption of the chlorophyll a (665 mμ) is recommended.

Some absorption at both these wave lengths is found even when all the chlorophyll has disappeared. This is due to the presence of yellow pigments which are not degraded as rapidly as chlorophyll.

The retention of chlorophyll bears a linear relation to the logarithm of the kinetin concentration over a range of approximately 0.05 to 5.0 μg. These values correspond to concentrations of 0.1 to 10 mg/l. The relationship is not linear for quantities of kinetin

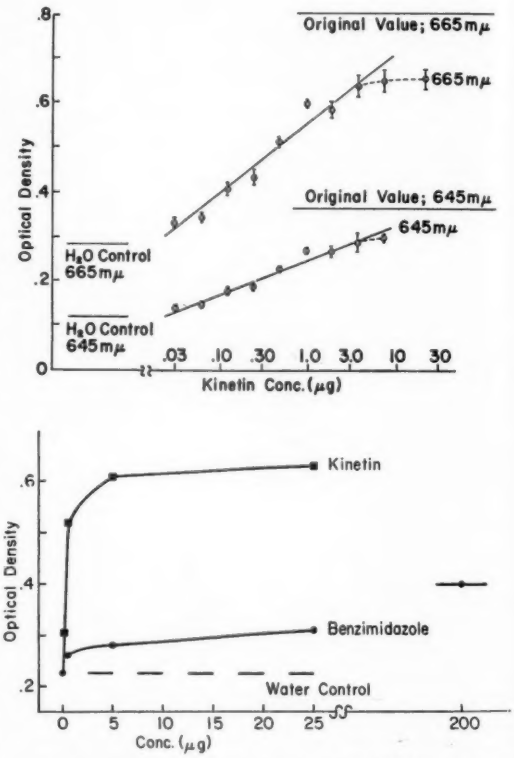

Fig. 1. Retention of chlorophyll by kinetin in discs of senescing Xanthium leaves. Optical densities of chlorophyll extracts in 80 % ethanol determined at the chlorophyll a and chlorophyll b maxima 48 hours after treatment.

Fig. 2. Comparison of the effectiveness of benzimidazole and kinetin in preventing loss of chlorophyll in senescing Xanthium leaf discs. Optical density of 80 % ethanol extract determined at 665 mμ.

above 5 μg because a maximum value for chlorophyll retention is obtained. Under these conditions the assay still provides a qualitative test for kinetin at least up to the maximum amount tested (25 μg).

6-Benzylaminopurine which is active in promoting cell divisions in tobacco pith cultures (9) is also active in the leaf disc assay, as is its riboside. Person et al (6) have shown that chlorophyll degradation in detached wheat leaves is prevented by benzimidazole as well as by kinetin. Figure 2 shows that with Xanthium leaf discs benzimidazole is not nearly so effective as kinetin.

A number of other compounds have been tested for possible interference in this assay. These include auxins (IAA & 2,4-D) and amino acids which are inactive, purines (adenine, adenosine, & guanosine) and 1,3-dimethylurea which interfere slightly but even at high concentrations give values lower than those obtained with 0.05 μg kinetin. Sugars in large amounts (2 mg) give values approaching those for 0.05 μg kinetin.

Discussion

This bioassay offers a rapid and simple screening test for material with kinetin-like properties. If desired, materials giving positive results in this assay could be tested using the tissue culture techniques. The leaf disc assay has several advantages. It does not require the use of complex media and sterile conditions and it gives quantitative results very rapidly.

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THIOCARBAMATES AS PLANT GROWTH REGULATORS[1, 2]

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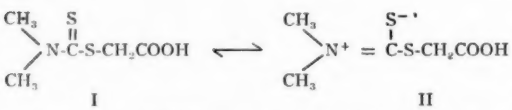
The findings of van der Kerk and his coworkers (10) that carboxymethyl dimethyldithiocarbamate (1) caused epinastic curvature and leaf deformations as well as some elongation of Avena coleoptile tissue related its action as a growth regulator to that of indole-3-acetic acid and other aromatic structures. However, there are several unusual aspects of this compound and its activity. It is the only such regulator without an unsaturated ring system. It is an extremely polar compound, whereas the other regulators have a large nonpolar structure as a part of the molecule. Only a very few of its analogues have activity. Its activity is shown only at relatively high concentrations.

In an attempt to rationalize the activity of the dithiocarbamates in terms used for the indoleacetic and phenoxyacetic acids by Veldstra (12), van der Kerk and his coworkers (10) suggested that the resonance in the thioamide group would tend to hold the two alkyl groups attached to the nitrogen in the same plane as the N-C(=S)- group which would also be planar and make this grouping of atoms comparable to the aromatic rings in the phenoxyacetic and indoleacetic acids.



I II

Further, the inactivity of dithiocarbamates with higher alkyl groups on the nitrogen atom was suggested to result from the lesser mesomeric electron release by the nitrogen atom and thus the lesser contribution of II to the structures. However, Chatt, Duncanson, and Venanzi (2) found no change in the contribution of II to the structures on ascending the homologous series.

Because of the relatively high concentrations of the dithiocarbamate required for activity, Gordon (4) investigated the possibility that the activity resulted from a sparing action that increases the amount of indoleacetic acid available to physiologically active sites. He concluded that neither a sparing action nor an acidity effect was responsible for the activity, but suggested the dithiocarbamate might act as a chelating agent and promote growth in the same way as ethylenediaminetetraacetic acid was found to do by Bennet-Clark (1). However, Fawcett, Wain, and Wightman (3) have pointed out that the chelating agents do not cause leaf epinasty or curvature of pea stems and thus the activity of the thiocarbamates is more similar to that of indoleacetic and phenoxyacetic acids.

In this investigation the possibility was explored that other N-substituted-dithiocarbamates might have growth promoting activity and thus provide some clue as to the mode of action of this structure. Since a great amount of evidence has been accumulated (12) in favor of the hydrophilic/lipophilic character of a compound as a factor in activity, attention was directed toward compounds with an increased lipophilic character.

MATERIALS & METHODS

Effects of the thiocarbamates on the elongation of Avena coleoptile tissue were measured with a standardized procedure (6) employing sections 3 mm in length. Because of the insolubility of the ethyl esters of the thiocarbamates in water, 2-propanol was used as required to dissolve the compounds. Fifteen sections were floated on the surface of 25 ml of the solution in a petri dish. Distilled water passed over mixtures of Amberlite ion-exchange resins was redistilled over glass before using. All solutions were at a pH of 5.5 to 5.8 initially and unbuffered. Length measurements were made after 24 hours except in tests of interaction with indoleacetic acid (IAA) where measurements were made after 12 hours.

The preparation of the sodium and ammonium dithiocarbamates was similar to the procedure reported by Heller (5). One-half mole of the amine was mixed with 150 ml of concentrated NH_4OH and 38 g of CS_2 were added. After shaking, the mixture was allowed to stand for 12 hours and the resulting precipitate was removed by filtration. This product was washed once with water, dried, and then used without further purification for the preparation of the ester derivatives of the thiocarbamates. The dithiocarbamates of the following amines were prepared in this fashion: dibutylamine, cyclohexylamine, piperadine, methylaniline, and morpholine. The sodium salt of the dithiocarbamate from dimethylamine was generously supplied by the DuPont Co. The ammonium salt of diethylamine was so soluble that it

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[1] Received October 3, 1960.

[2] This research was supported in part by a grant from the Research Corp. to Pomona College.

was found to be more convenient to prepare the sodium salt. The procedure was the same except that 200 ml of 50 % sodium hydroxide were used in place of ammonium hydroxide.

In the preparation of the ethoxycarbonylmethyl dithiocarbamates, typically 0.10 mole of the ammonium salt was dissolved in 40 ml of 50 % aqueous ethanol and 9 g of ethyl bromoacetate were added. The mixture was placed on a shaker over night. In some instances part of the ester separated either as a liquid or a solid. The solution was diluted with water and the ester separated. The solid esters were then purified by recrystallization from methanol. The diethyl derivative did not solidify until it had been flash-distilled under vacuum. The dibutyl derivative did not solidify even after vacuum distillation. Ethoxycarbonylmethyl diethyldithiocarbamate, ethoxycarbonylmethyl dibutyldithiocarbamate and ethoxycarbonylmethyl dithiocyclohexanecarbamate have not previously been reported in the literature and analytical data in support of the structures are given in table I.

Results & Discussion

Although 2-propanol has been used to facilitate the dissolving of nonpolar compounds in aqueous solutions in several investigations, (7, 11) no intrinsic effect on the growth response of coleoptile tissue was examined. A significant effect of 2-propanol was observed in the comparison of the effect of the ethyl ester of the dithiocarbamate structure with the acid. As shown in figure 1, 0.5 % 2-propanol reduces the growth response of coleoptile tissue to indoleacetic acid by 16 to 28 %. Similarly 2 % 2-propanol has been found to reduce the growth response in water by 45 % and in IAA by 30 to 50 %. Thus the lesser effect of the ethyl ester at 5×10^{-5} M as shown in figure 1 may be attributed to the effect of 2-propanol and the greater growth induced by the ester at 5×10^{-6} M has additional significance. The polarity of the dithiocarbamate is reduced considerably by transformation to the ethyl ester and its activity in growth is thereby increased. The small growth obtained at 5×10^{-4} M, then reflects an interference in the growth reaction by high concentrations of the ester.

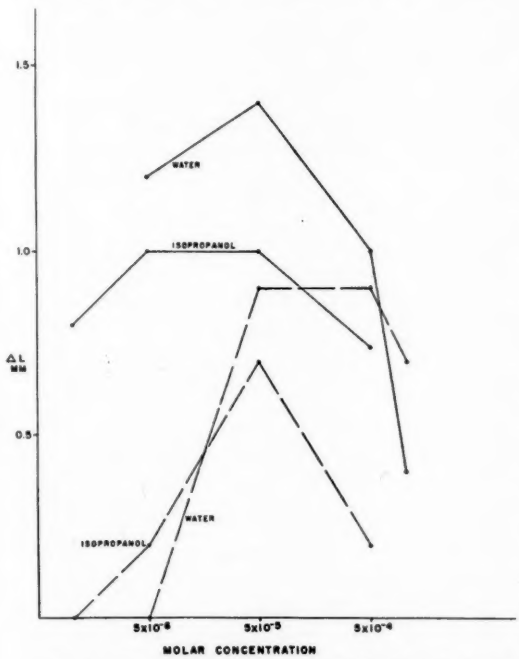


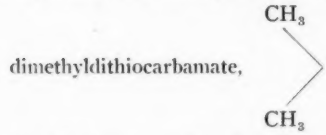
Fig. 1. Growth as increment of length of Avena coleoptile sections after 24 hours induced by indole-3-acetic acid (*solid lines*) in water and in 0.5 % 2-propanol, and carboxymethyl dimethyldithiocarbamate in water and its ethyl ester in 0.5 % 2-propanol (*broken lines*).

The effects of the ethyl esters of seven N-substituted carboxymethyl dithiocarbamates on the elongation of coleoptile tissue are given in table I. The percentage stimulation was arrived at by comparison with the elongation taking place in 2-propanol which usually would be about 0.5 mm. If the elongation in the solution were 1.0 mm then the percentage stimulation would be $\frac{1.0 - 0.5}{0.5} \times 100$ or 100 %. Only the dimethyl and diethyl derivatives were found to promote elongation with the diethyl derivative much less effective. This finding is in agreement with the original report of van der Kerk et al (10) that "ethyl or higher alkyl groups" on the nitrogen atom resulted in "slight activity or inactivity" for the acid forms. Fawcett, Wain, and Wightman (3) have also reported on the elongation effect of the diethyl derivative of the acid, finding it with about the same activity as the dimethyl form at approximately 5×10^{-4} M but with lesser effect at 5×10^{-3} M. The ethyl ester form here also has an effect at the low concentration of 5×10^{-5} M where the acid form has no effect (3).

Substitution of two butyl groups on the nitrogen atom not only eliminates any stimulatory effect on elongation but brings about strong inhibitory effects. Similarly hexanyl substitution or incorporation of the nitrogen atom into piperidine or morpholine rings give inhibition of elongation.

The suggestion of van der Kerk and coworkers (10) that the grouping of atoms around the nitrogen atom would be comparable to an aromatic ring is difficult to accept since its high degree of polarization would be in no way comparable to a benzene ring either from the point of view of solubility or adsorbability. To examine the importance of the planar configuration, ethoxycarbonylmethyl N-methyldithiocarbanilate was synthesized. In this molecule the flat benzene ring and its possibility for resonance with the lone pair of electrons of the nitrogen should increase the trigonal character of the nitrogen atom and thus aid in preserving the flatness of the thioamide group as well as the two groups attached to the nitrogen. This molecule might be compared

roughly (using van der Kerk's model) to indoleacetic acid. The complete absence of a stimulatory effect of this compound leads us to doubt the importance of the planar character of the thioamide group for biological activity. Furthermore, if planarity were all important, then the oxygen analogue of carboxymethyl



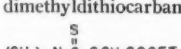
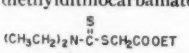
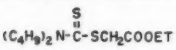
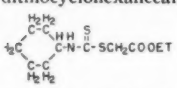
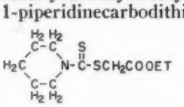
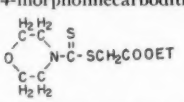
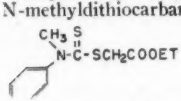
dimethyldithiocarbamate,

should be more active since the electrons of the oxygen can overlap the *p* orbitals of the carbon atom more effectively than sulfur. Thus one would expect more trigonal character to the nitrogen atom. However, the oxygen analogue has insignificant activity (3, 10, 13). If, as Veldstra (12) has emphasized, a non-polar ring ordinarily constitutes the planar structure of an active molecule, it is difficult to reconcile the substitution of the phenyl ring in the dithiocarbamate molecule and its inactivity. Substitution of the methyl and phenyl groups on the nitrogen atom is not inactivating per se since N-methyl—N-phenylglycine and its 4-chloro- and 3,4-dichloro- amide derivatives have been shown to be active (8, 9).

The strong inhibitory effect on elongation found for the ethoxycarbonylmethyl N-methyldithiocarbanilate led to an examination of its interaction with indoleacetic acid. In figure 2 the reciprocal plots of elongation and concentration of indoleacetic acid in the presence of both the dithiocarbanilate and the ethoxycarbonylmethyl dimethyldithiocarbamate and 0.5 % 2-propanol are shown. The effect of the dithiocarbanilate is that of a competitive inhibitor of the growth induced by indoleacetic acid. The stimula-

TABLE I

CHEMICAL & BIOLOGICAL PROPERTIES OF ETHYL ESTERS OF DITHIOCARBAMATES & RELATED STRUCTURES

COMPOUND	MELTING POINT	CARBON-HYDROGEN ANALYSIS CALCULATED		ANALYSIS FOUND		% 2-PROPANOL	% STIMULATION (+) OR INHIBITION (−) OF ELONGATION					
		% C	% H	% C	% H		10^{-6}	5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4} M
1. Ethoxycarbonylmethyl dimethyldithiocarbamate 	55–56° C					0.5	0	+40		+135		+40
2. Ethoxycarbonylmethyl diethyldithiocarbamate 	51–52° C	45.93	7.28	46.28	7.28	1.0		0	0	+45	+45	−60
3. Ethoxycarbonylmethyl dibutyldithiocarbamate 	b.p. 152–57° C/10mm	53.57	8.65	53.46	8.45	2.0		−20	−40	−100	−100	
4. Ethoxycarbonylmethyl dithiocyclohexanecarbamate 	77–79° C	50.54	7.33	50.66	7.90	0.5	0	0	−20	−35		
5. Ethoxycarbonylmethyl 1-piperidinecarbodithioate 	74–75° C					1.0		0	0	−20	−35	
6. Ethoxycarbonylmethyl 4-morpholinecarbodithioate 	56–57° C					0.5	0	−20	−20	−25		
7. Ethoxycarbonylmethyl N-methyldithiocarbanilate 	71–73° C					1.0		−10	−10	−50	−50	

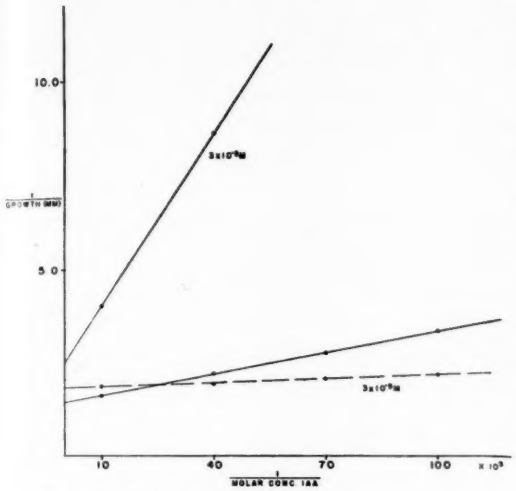


FIG. 2. The reciprocal of growth rate of Avena coleoptile sections plotted as a reciprocal function of the concentration of indole-3-acetic alone and in the presence of 3×10^{-5} M ethoxycarbonylmethyl N-methyl-dithiocarbanilate (*above*) and ethoxycarbonylmethyl dimethyldithiocarbamate (*below*).

tory effect of the dithiocarbamate is apparently additive at the lower concentrations of indoleacetic acid but completely nullified at higher concentrations. It thus appears that these substances and indoleacetic acid have at least one reaction site in common, that reacting with the carboxyl group. It also is probable that the effect of the dithiocarbamate involves a reaction of the electron-rich nitrogen atom. The activity of the nitrogen would be reduced by any substituent capable of delocalizing the lone pair of electrons or blocked by groups larger than ethyl.

The action of the dithiocarbamates by means of a chelating mechanism involving the carboxyl group and the sulfur or nitrogen atom is unlikely since both ethoxycarbonylmethyl 1-piperidinecarbodithioate and ethoxycarbonylmethyl 4-morpholinecarbodithioate are incapable of stimulating elongation. In these the groups attached to the nitrogen would not provide enough steric hindrance to destroy any chelating ability they might have. At present there is no evidence to indicate that the dithiocarbamates act in a manner different from indoleacetic acid.

SUMMARY

Among seven ethyl esters of N-substituted carboxymethyl dithiocarbamates only the dimethyl and diethyl derivatives were active in promoting elongation of Avena coleoptile tissue. 2-Propanol at 0.5% required to dissolve the ethyl esters was found to repress the intrinsic elongation of the tissue as well as that induced by indoleacetic acid. Thus less growth is obtained with the ethyl ester of carboxymethyl dimethyldithiocarbamate than with the acid at high concentrations but at 5×10^{-6} M the ester has an effect while the acid has none. Reduction of the polarity of the dithiocarbamate by transformation to the ethyl ester increases its activity in elongation.

Substitution of two butyl groups or methyl and phenyl groups on the nitrogen atom results in compounds with strong inhibitory action on elongation. Inhibition also occurs with hexanyl substitution or incorporation of the nitrogen atom into piperidine or morpholine rings. The planar character of the thioamide group does not appear to be a factor in the biological activity of these compounds, nor does it appear that a chelating mechanism is operative.

The interaction of the dimethyldithiocarbamate and ethoxycarbonylmethyl N-methyldithiocarbanilate with indoleacetic acid suggests that these substances have at least one reaction site in common, that reacting with the carboxyl group. It also is probable that a reaction of the electron-rich nitrogen atom is involved.

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A NATURALLY OCCURRING CHELATE OF IRON IN XYLEM EXUDATE[1,2]

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At the normal pH of plant tissues, iron forms extremely insoluble hydroxide and phosphate precipitates. The K_{sp} of iron phosphate has been reported to be of the order of 10^{-35} over a relatively wide pH range (5). The form in which iron is transported under these conditions becomes of particular interest, for if inorganic iron and phosphate are both present in the xylem stream, it would seem that iron would be present in such insoluble precipitates that the amounts moving from the roots to the shoots would be extremely small, and iron deficiency would develop in the leaves and stems.

Biddulph and Woodbridge (1) detected the presence of a precipitate of iron-phosphate on the root surfaces of plants grown in nutrient cultures with high phosphate content. They proposed that iron also may be precipitated in this way in the xylem channels thus causing a deficiency of iron in the plant. Olsen (9) suggested that iron-phosphate precipitation may lead to deficiency symptoms in plants which seem to have a normal iron content. Since under normal growing conditions plants do not show iron deficiency, the occurrence of such blocking precipitates seems doubtful.

Deficiency symptoms attributable to genetic variation have been reported in grapes (22) and in soybeans (23). In these cases, some variants developed deficiency symptoms in nutrient media in which other variants made normal growth. Brown and Tiffin (3) found differences in the iron content of exudate from the soybeans, and proposed that certain genetic variants are not able to absorb sufficient iron from the nutrient medium.

The present investigation was carried out to determine if a natural complex or chelate of iron which can prevent formation of the inorganic precipitates exists in plants and serves as a transport form for iron. Several workers in recent years have suggested this possibility. Burstrom and Tullin (4) proposed that some of the many organic materials found in xylem fluid may function as chelaters of metals and Stewart and Leonard (20) suggested that chelates are the natural form in which iron is absorbed from the soil by higher plants. The classes of organic compounds present in grape vine exudate have been studied by Priestley and Wormall (11), and in a variety of other plants by other workers.

Because of the difficulty of obtaining xylem sap in the required quantities directly from plant stems, the work to be reported here will include only studies on tobacco plant exudate.

MATERIALS & METHODS

Exudate was collected from the stumps of tobacco plants (*Nicotiana tabacum* L. var. Wisconsin 38) according to the method described by Grossenbacher (7). It normally was collected for approximately six hours after cutting and used immediately. When not used immediately, the exudate was kept under refrigeration.

Total iron content of the exudate was determined by a potassium thiocyanate method using amyl alcohol as an extracting medium and hydrochloric acid for acidification (15). For certain analyses, other reagents also were used. These included Tiron (24), chromotropic acid (3,6-disulfo-1,8-dihydroxynaphthalene), Bathophenanthroline (19), and acetylacetone (12). In the latter case, it was found that the iron-acetylacetone colored complex could be extracted into amyl alcohol. This considerably increased the sensitivity of the test.

Several time-color development tests were carried out employing reagents which form colored complexes with iron (Tiron & chromotropic acid). Because of the relatively low sensitivity of these reagents, color intensities were determined in a Klett-Summerson Colorimeter using a cell which provided a 4 cm light path.

Phosphorus was determined by a stannous chloride-reduced phosphomolybdate method (8) and nitrogen by a semi-micro Kjeldahl procedure. Ascorbic acid was analysed by the method of Roe et al (14).

Cation and anion exchange resins were used in both column and batch tests. Dowex-1 was prepared in the acetate form by treatment with sodium acetate and acetic acid; Amberlite IR-120, in the sodium form by treatment with sodium chloride and hydrochloric acid. Small resin columns were prepared by attaching a 1.0 cm I.D. glass tube (14 cm long) to the center of the base of a 125 ml Erlenmeyer flask. A constriction in the tube about three centimeters from the lower end served as a support for a wad of glass wool on which the resin bed was supported. The flask served as a reservoir for eluent.

Large resin columns were made from glass tubing

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[2] This investigation was supported in part by the University Research Committee with funds from the Wisconsin Alumni Research Foundation.

2.5 cm I.D. and 37 cm long. At the lower end, a fritted glass plate was inserted to serve as a base for the resin bed. For ease in collecting fractions, the lower end of the column was constricted to a small spout. After passing exudate through the large resin columns, they were eluted with 0.25 N HCl and fractions of 10 ml volume were collected with a Gilson automatic fraction collector. Every third fraction was tested for iron content.

In batch work with resins, exudate samples were in contact with the resin in an Erlenmeyer flask for 24 hours under refrigeration and with occasional stirring.

Exudate was also fractionated with Sephadex G-25, a gel filter (10). The large column and the fraction collector used were as described for the ion exchange resin.

Paper chromatograms were prepared with Whatman no. 1 paper cut into rectangles 7.5 by 9 inches. Before use, the paper was washed in dilute hydrochloric acid followed by at least five washings in distilled water. This treatment significantly reduced iron contamination. Separation of amino acids was by the method of Sisakyan et al (17) with *n*-butanol, methyl ethyl ketone, water (2/2/1) as the solvent system. Diethylamine, which they also used, was not included in the solvent for the present work. Amino acid spots were detected by ninhydrin spray reagent.

In further attempts to isolate the iron containing fraction in the exudate, a mixture similar to the solvent system published by Chu and Chu (6) for separation of iron containing porphyrins was employed. The developing solvent consisted of isopropanol, water, pyridine (0.1/5.5/0.4).

Bathophenanthroline, an extremely sensitive iron reagent, was employed to locate iron-containing spots on the chromatograms. The paper was dried at room temperature after development, sprayed with 10 % hydroxylamine hydrochloride, dried at 70° C, and sprayed with 0.03 % bathophenanthroline reagent prepared according to Smith et al (19). On further drying at 70° C, the presence of iron was indicated by the appearance of pink spots.

All filtration of iron containing exudates or solutions in the present work was through a Millipore filter, size HA (0.45 μ pore size.)

Results

Total Iron, Nitrogen, & Phosphorus Contents of Exudate: In most cases the iron content of the exudate was in the range of 1.0 to 1.3 μg/ml (ppm), although some exudates contained as little as 0.4 μg/ml and the maximum was 1.7. Exudation occurred in a diurnally periodic pattern similar to that reported by Skoog et al (18) in sunflowers, but the iron content of the exudate gradually fell with time after cutting and showed no periodicity.

Phosphorus content varied from 50 to 70 μg/ml with one determination showing over 100 μg/ml. Nitrogen was present in concentrations of 60 to 75 μg/ml. The pH of the exudate was approximately 5.5.

Removal of Iron From Exudate by Millipore Filtration: To ascertain whether or not the exudate iron was present in a precipitated form, exudate was passed through a Millipore filter and the iron content of the filtrate was compared with that of the original exudate. In no case was the filtrate iron content reduced by this treatment.

At various times, batches of exudate were collected, held in the refrigerator for different lengths of time up to 3 days, and then tested for iron before and after Millipore filtration. The results are presented in figure 1 in which per cent of total iron remaining after Millipore filtration is plotted as a function of time from collection. It may be seen from the upper curve that the iron remains quite soluble even after 3 days. The slight removal with time may be due to bacterial action leading to breakdown of an iron complex. Within 2 days, the exudate becomes quite turbid and a precipitate settles to the bottom, but even then the amount of iron still complexed in solution has changed only slightly.

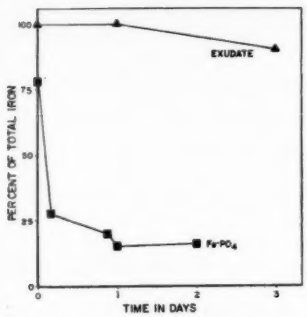
For purposes of comparison, a pH 5.0, acetate-buffered solution containing 1 μg of inorganic iron and 50 μg of inorganic phosphorus per milliliter was prepared and aliquots were passed through a Millipore filter at the time intervals indicated in figure 1. It may be seen (lower curve) that after only 4 hours, Millipore filtration removed a large percentage of the iron from solution. These results are in agreement with those of Rediske and Biddulph (13) and in contrast with the behavior of iron in exudate.

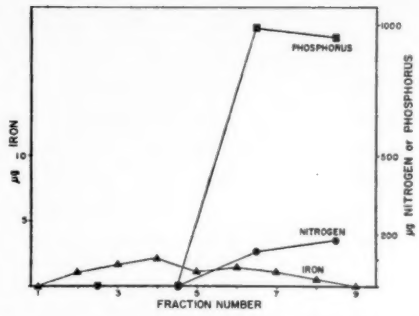
Studies With Ion Exchange Resins: The possible presence of iron as a free cation in the exudate was checked through the use of a cation exchange resin. A batch test was made with Amberlite IR-120 and the supernate was analysed for iron. In the several tests made, there was no reduction in iron content of the supernatant liquid indicating that exudate iron is not in a form which can be removed by a cation exchange resin.

Amberlite IR-120 was capable of removing ionic iron from an acetate-buffered solution containing ferric nitrate. After iron had been fixed onto the resin from the acetate-buffered solution, it then could be removed by chromotropic acid, indicating that the resin's affinity constant (log K) for iron is less than 17.0 (that of chromotropic acid).

In further investigations, all the iron was removed from samples of exudate passed through a small column of an anion exchange resin, Dowex-1. This indicated that exudate iron was present in an anionic form. To eliminate the possibility of a filtering action by the resin bed, a batch test also was carried out with this resin. After 24 hours all iron again had been removed from the supernate.

It also was found that after exudate had been passed through a Dowex-1 column and the column





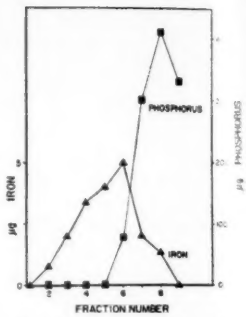


FIG. 1. Iron present in Millipore filtrates of tobacco plant exudate and in a synthetic solution containing iron and phosphate filtered at various intervals after exudate collection or solution preparation.

FIG. 2a. Iron, phosphorus, and nitrogen contents of successive samples from a Sephadex fractionation of tobacco exudate.

FIG. 2b. Iron and phosphorus contents of successive samples from a Sephadex fractionation of tobacco exudate.

had been washed with distilled water, it was capable of removing additional iron (1 μg/ml) from an acetate-buffered solution. This suggested that a compound which had become bound to the anion column was capable of tying up more iron than was originally present in the exudate.

After trials with various eluting agents, 0.25 N HCl solution was found suitable for washing the exudate iron from a Dowex-1 column on which it had been fixed. The fractions having a high iron content were pooled, neutralized to pH 5.0 with dilute ammonia, mixed with Dowex-1 in a test tube, and allowed to sit with occasional stirring in the refrigerator for about 48 hours. A test for iron revealed that all the iron again had been taken up by the resin.

PAPER CHROMATOGRAPHY OF EXUDATE: The possible existence of an iron complex also was investigated by chromatographic separation of the organic materials in exudate. Chelation with an amino acid was suggested by the work of Bollard (2) on the amino acids in xylem sap of apple trees. Tobacco exudate was chromatographed with the solvent of Sisakyan et al (17) and following spraying with Ninhydrin several amino acid spots were observed. The largest spot was shown to be glutamine; the smaller spots were not identified. No movement of exudate iron occurred although the Ninhydrin spots were all away from the origin. No Ninhydrin color was visible at the origin. A spot of ferric nitrate standard on the same chromatogram also failed to move from the origin.

A mixture of iron and glutamine was chromatographed with the above solvent. The iron again failed to move, thus apparently discounting the possible function of this amino acid as the iron complexing agent in exudate.

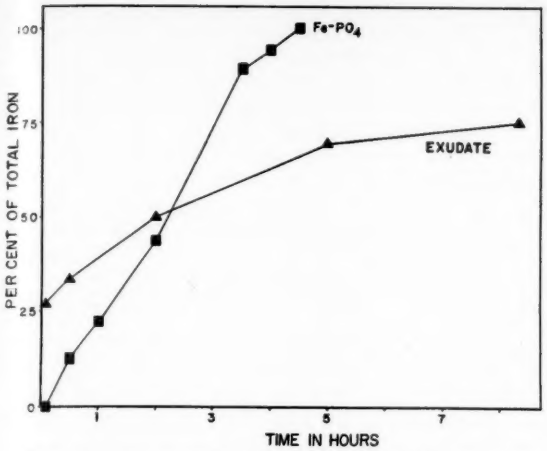
Further attempts were made to find a solvent which could move exudate iron. Development of chromatograms with butanol-acetic acid-water solvent, a standard method for separation of organic acids which are known to be present in exudate, was not successful in moving iron. The solvent used by Chu and Chu (6) to separate iron-containing porphyrins moved exudate iron from the origin to a point near the solvent front. However, iron from a ferric nitrate standard as well as from an iron-phosphate mixture did not move.

During the course of investigations with Dowex-1 columns, it had been noted that on passing exudate through a column a dark-brown layer was deposited at the top of the column. This layer moved downward on elution with dilute hydrochloric acid. Furthermore, exudate always had a slight yellow color. Shannon (16), in the course of studies on iron chlorosis, employed ascorbic acid as a chelating agent, and this was considered as a possible explanation for the color in the resin column and as the natural chelating agent. Initial results were promising, for when oxidized ascorbic acid was run through a Dowex-1 column, a brown layer formed at the top and if acetate-buffered iron solution were then passed through the same column, the iron was retained.

Furthermore, a buffered solution of iron and oxidized ascorbic acid was successfully chromatographed with the isopropanol-pyridine-water solvent (6) and the iron was moved to the same degree as in exudate spots.

However, in a test of the dialysibility of exudate iron, it was found that very little iron moved out of the membrane whereas in the case of iron-ascorbate, iron was readily moved through the membrane. Exudate contained about 15 μg of ascorbic acid per ml.

GEL FILTRATION COLUMN: At this time, Sephadex (a gel filter used to separate relatively large molecular weight substances from solution) was introduced as an additional means of purifying the exudate fraction containing iron. Millipore-filtered exudate was passed through a column of Sephadex and the resulting fractions analysed for iron, nitrogen, and phosphorus. The results are presented in figures



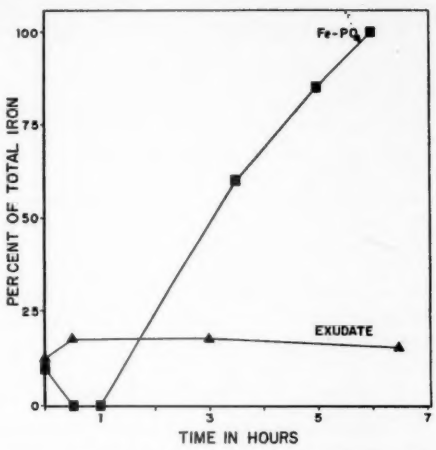


FIG. 3a. The capacity of Tiron (log K=20.7) to complex iron over a period of time from tobacco exudate and from a solution containing inorganic iron and phosphate.

FIG. 3b. The capacity of chromotropic acid (log K=17) to complex iron over a period of time from tobacco plant exudate and from a solution containing inorganic iron and phosphate.

2a and 2b. For the data of figure 2a, successive pairs of fractions were combined and analysed for phosphorus and nitrogen after individual fractions had been analysed for iron. The results with phosphorus led to a further test in which the phosphorus content of each sample from a Sephadex fractionation was determined and compared with the iron content (fig 2b). The separation of iron from both nitrogen and phosphorus strongly indicated that the iron complex was not a compound containing nitrogen or phosphorus and that the iron was associated with a relatively large molecule. Tests for the amino acid content of fractions from a smaller gel filter column had shown that no amino acids occur in the fractions containing iron, thus substantiating the results with paper chromatography of amino acids. The fact that phosphorus was separated from the iron also corroborates results with paper chromatography and Millipore filtration. A mixture of iron-ascorbate was retained by the gel filter against elution, thus further discounting this compound as a possible complexing agent.

COMPLEXING OF EXUDATE IRON: In order to more firmly establish the approximate affinity constant of exudate for iron, the capacity of several commercially available chelating agents which form colored complexes with ferric iron to complex with iron in exudate was determined. Results with three of these are presented in table I together with the percentage of the total iron which they indicated to be present in the exudate. These results were obtained with the colorimeter immediately after mixing the exudate and the complexing agents. It is obvious that in no case did these reagents indicate the true amount of iron present in the samples, thus strongly suggesting the presence of a material which was holding the exudate iron and not readily releasing it to the reagents used.

The capacities of Tiron and chromotropic acid to complex with iron in exudate and in a synthetic iron-phosphate mixture were then determined over a period of time. The time-color development curves for these studies are presented in figures 3a and 3b. The values on the ordinate axes are derived from determinations corrected for an exudate blank and for a reagent blank.

In figure 3a, the behavior of exudate and of the iron-phosphate mixture with Tiron is shown. While the removal of iron from the iron-phosphate mixture is linear with time, the curve for removal from exudate would have to be plotted in a logarithmic fashion in order to obtain a straight line. It is to be noted that the two curves indicate a very different behavior

TABLE I

COMPARISON OF CAPACITY OF VARIOUS COMPOUNDS WHICH FORM COLORED COMPLEXES WITH FERRIC IRON TO COMPLEX WITH IRON IN XYLEM EXUDATE*

REAGENT	IRON CONTENT, μg/ml	% OF TOTAL IRON COMPLEXED
KSCN	1.3	
Tiron (20.7)	0.3	23
KSCN	0.5	
Acetylacetone (9.0)	0.0	0
KSCN	1.0	
Chromotropic acid (17)	0.2	20

* Affinity constants (Log K) are given in parentheses.

of iron from the two sources, and that after eight hours Tiron had complexed 75 % of the total exudate iron.

Figure 3b presents results of a time-color development study with chromotropic acid. The reaction with the iron from the iron-phosphate mixture is again linear and occurs quickly while not more than 20 % of the exudate iron reacted with chromotropic acid. At 24 hours it had risen somewhat, but a spurious orange color had formed and the results are reported to 9 hours only.

The fact that these two reagents seem to bracket the affinity of exudate for iron suggests that the stability constant of the natural complexing agent and iron is between 17 and 20.7.

Discussion

Although recent literature on iron nutrition contains suggestions that a natural chelate functions in iron transport in plants (21), to the authors' knowledge there are no published results verifying this proposal or identifying the complex. In the present investigation, evidence has been presented for the existence of an iron-containing complex in exudate, primarily through demonstrations that in a variety of tests exudate iron behaves differently than if it were present as free iron or as an insoluble precipitate. Furthermore, treatment with ion exchange resins revealed that exudate iron is associated with a complex which behaves as an anion.

The existence in the xylem of a precipitate containing iron seems to have been eliminated by filtration studies. In no case was iron removed from exudate by Millipore filtration. Even in view of this, it might still be argued that exudate iron is present as a very finely divided phosphate precipitate which could pass through the filter. This seems to be negated by the fact that in a synthetic iron-phosphate mixture, prepared to approximate the phosphorus and iron contents of exudate, iron immediately began precipitating in a form which could be Millipore filtered. Furthermore, paper chromatographic evidence showed that, in contrast with exudate iron, iron in a synthetic mixture does not move with the solvents employed.

The data presented include evidence on the approximate affinity constant between iron and the exudate complexing agent. This was determined to be between 17 and 20.7 by the differential capacity of Tiron and chromotropic acid to complex with exudate iron. Both of the complexing agents were capable of removing iron from the iron-phosphate mixture but only Tiron could remove iron from exudate. The affinity of the complexer need not be as great as might be anticipated, since once iron has been complexed it apparently is not readily released even for the formation of very insoluble precipitates. For example, acetate ion is capable of holding iron in solution at pH 5, although it releases iron to Amberlite IR-120 cation exchange resin.

Identification of the iron complexing agent in plant exudate must await further studies. In the present investigation, amino acids, organic acids, and ascorbic acid have been demonstrated to be present in exudate. However, they seem to have been eliminated as the possible chelating agent on the basis of behavior in several tests. Paper chromatography eliminated amino acids and results with gel filtration showed that nitrogen was not associated with the iron. The possibility that ascorbic acid or a related compound may function as a complexer was discounted by the fact that in a gel filter iron in an iron-ascorbate complex behaves differently from exudate iron.

It is of course obvious that an iron complex present in xylem exudate may not be present in the xylem stream of a transpiring plant and may not be the transport form of iron under these conditions. As mentioned earlier, the difficulty of obtaining adequate quantities of xylem sap led to the use of tobacco exudate. Following identification of the iron complex in exudate, it is hoped that this work can be extended to studies on sap collected from transpiring plants.

Summary

Since iron forms extremely insoluble phosphate and hydroxide precipitates at the pHs encountered in plants, studies were carried out to determine if a natural complexing agent is present in plant sap which prevents iron precipitation and serves as a transport form of this element. This was investigated primarily through comparisons of the behavior of iron in tobacco plant exudate and in synthetic solutions containing comparable quantities of inorganic iron and phosphate.

Millipore filtration did not reduce the iron content of exudate but almost completely removed iron from the synthetic iron-phosphate mixture. Exudate iron also moved on paper chromatograms; iron from the synthetic iron-phosphate mixture did not.

Exchange resins, chromatography, and gel filtration were employed to separate the proposed natural iron complex as an initial step in its identification. Iron was not removed from tobacco exudate by cation exchange resins, but was adsorbed onto an anion exchange resin from which it subsequently could be leached and reattached to the same resin. This suggests that the iron was associated with an anionic structure. Exudate iron moved directly through a Sephadex gel filter, suggesting it is associated with a relatively large molecule. A Sephadex column also separated the iron from nitrogen and phosphorus containing compounds. While these results strongly suggest the existence of a naturally occurring iron complexing agent which can prevent precipitation, its identity has not been established.

Paper chromatographic separations carried out thus far have indicated that neither amino acids, organic acids, nor ascorbic acid is the natural complexing agent in exudate.

The stability constant (log K) of the exudate iron complex was found to lie between 17.0 and 20.7 through a comparison of the capacities of synthetic chelating agents having various affinities for iron to remove iron from the exudate complex.

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TIME COURSE OF OXYGEN EVOLUTION DURING PHOTOSYNTHESIS IN SYNCHRONIZED CULTURES OF ALGAE [1, 2, 3]

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The time course of photosynthetic gas exchange for synchronized algal cultures in an acid medium has been shown to depend on the developmental status of cells (13, 14) and on light intensity (15, 17). In phosphate buffer, at pH 4.5, the rate of gas exchange in younger cells gradually increased with the progress in a photosynthetic experiment; that in older cells constantly decreased with time. In cells of intermediate age a peculiar phenomenon was observed. The rate of gas exchange first decreased but later recovered to a higher level. As the result of this recovery the rate in some experiments reached or even surpassed the level of the initial rate as measured at the start of the experiment. Both the downward and the upward trend in the time course of the gas exchange were shown to depend on light intensity. The downward trend was intensified by increase in light intensity. The upward trend was first favored by increase in light intensity, then, after light intensity reached saturation level and the light intensity curve for the upward trend formed a plateau, the curve bent down with further increase in light intensity.

In the work reported here, previous studies (13, 14, 15, 17) were extended to include the effects of light intensity on synchronized algal cultures in an alkaline medium. Carbonate-bicarbonate buffers have been extensively employed in kinetic studies of photosynthesis by many investigators. Their advantages and limitations in studies on synchronized populations are evaluated in the further discussion.

MATERIAL & METHODS

The work was done with the high-temperature strain, 7–11–05, of *Chlorella pyrenoidosa* Chick (12). The technique of growing synchronized algal suspensions was described in a previous publication (13).

To prepare algal suspensions for photosynthetic measurements cells in a predetermined developmental stage were harvested from the growth chamber and centrifuged for 30 minutes at 1,000 *g*. Then, without washing, cells were transferred into Warburg's carbonate-bicarbonate buffer no. 9 at pH 9.35. The buffer had been made by mixing 0.1 M solutions of bicarbonate and carbonate in the proportion 85 parts of bicarbonate to 15 parts of carbonate. Each of these consisted of equimolar concentrations of potassium and sodium salts. The amount of cells per 4 cc of buffer placed in one reaction vessel did not exceed 0.72 mm^3 of wet packed cells. Due to the small amount of cells per flask and to a potentially large reservoir of carbon dioxide in no. 9 buffer the pH of the medium did not change appreciably in the course of the experiments. The algal suspension was pipetted into rectangular reaction vessels and the vessels were placed in the bath thermostated at 39° C. To allow for temperature adjustment readings were not started until flasks had been shaken at a given light intensity for 10 minutes. In the following graphs the zero point corresponds, therefore, to the 10-minute reading.

Light was provided by a bank of tungsten lamps placed under the glass bottom of the bath. Light intensity was measured at the level of the reaction vessels and was expressed in foot-candles. Different levels of illuminance during photosynthetic measurements were achieved by attaching neutral filters of known transmittancy to the bottoms of the reaction vessels. The light intensities to which cells in different developmental stages were exposed during photosynthetic measurements are given in table I. In the following discussion the performances of the cells of different developmental stages are compared at approximately the same light intensity. The approximate intensity as represented in the graphs is indicated in the last line of table I.

Determinations of packed cell volume were made parallel to photosynthetic measurements by centrifuging known volumes of the original algal suspensions in calibrated capillary tubes. Pressure changes, as obtained from manometric readings, were converted

TABLE I

LIGHT INTENSITIES TO WHICH CELLS IN DIFFERENT DEVELOPMENTAL STAGES WERE EXPOSED DURING PHOTOSYNTHETIC MEASUREMENTS

DEVELOPMENTAL STAGE OF CELLS, hr	LIGHT INTENSITIES, ft-c
0	215 ... ... 1,525 2,030 ...
4	197 474 778 1,502 1,984 2,162
6	223 ... ... 1,552 2,079 ...
Nearest approximation	200 1,500 2,000

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[2] This work was supported in part by funds from the National Aeronautics and Space Administration.

[3] Scientific Article A 881, Contribution no. 3193 of the University of Maryland Agricultural Experiment Station.

into volume changes and expressed in mm^3 O_2 evolved per mm^3 of packed cells from the start of measurements till the time indicated on the graphs (fig 1, 3, 4, & 5). To indicate absolute (fig 2) or relative rates (figs 6 & 7) oxygen evolution was calculated per 1 hour. No correction for respiration was made.

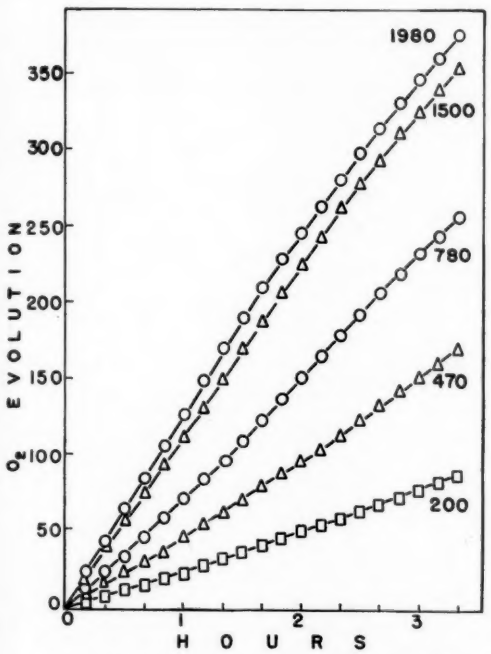
Results

The family of curves presented in figure 1 describes the time course of oxygen evolution in 4-hour cells with light intensity as a parameter. The curve obtained at 2,160 ft-c was omitted from the figure. It actually ran between the curves obtained at 1,500 and 1,980 ft-c and was very close to the second of these.

The initial rate of oxygen production, measured for the first hour of observation, was favored by increase in light intensity up to about 2,000 ft-c. With progress in the photosynthetic experiment different light intensities exerted different effects on the time course of gas exchange. At 200 ft-c, the rate gradually and slowly increased during the whole 200 minutes of observation. At 470 ft-c, the initial rate and the increase with time were greater than at 200 ft-c. At 780 ft-c, the rate increased for about one hour. Then, for about 100 minutes (from 60–160 min), it remained steady and after that time it began to decline. At 1,500 and 1,980 ft-c, there was no increase in the rate with time and the decline in the rate began earlier than at 780 ft-c.

As judged by the time at which the rate of gas production begins to decline, the increase in light intensity is eventually detrimental to photosynthetic activity. At 200 and 470 ft-c no decline in the rate was recorded during 200 minutes of observation. At 780 ft-c a decline was recorded after 160 minutes; at 1,500 ft-c, after 140 minutes; and at 1,980 ft-c, after 100 minutes.

The effects of the time factor and of light intensity on the course of oxygen evolution are shown in figure 2, in which the photosynthetic rates measured for the 1st, 2nd, and 3rd consecutive hours of observation are plotted against light intensity. The shape of the light intensity curve varied according to the period for which observations were made. At lower light intensities, rates were higher at later times of observation. At higher light intensities, rates were much lower if measured later in the course of the experiment. Data obtained for the 1st hour of observation produced a curve with light saturation at about 2,000 ft-c and maximum saturation rate of



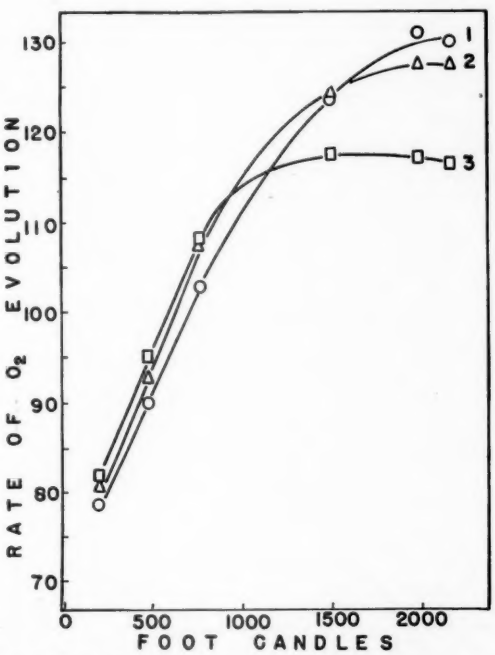


FIG. 1 (*left*). Time course of oxygen evolution in 4-hour cells of Chlorella 7–11–05. Oxygen evolution (apparent photosynthesis) in volumes of gas per packed volume of cells. Light intensities indicated in foot-candles on the curves.

FIG. 2 (*right*). Rates of oxygen evolution in 4-hour cells of Chlorella 7–11–05 at different light intensities. Oxygen evolution (apparent photosynthesis) in volumes of gas per packed volume of cells per hour. The rates were computed separately for each of the three successive hour intervals of each photosynthetic experiment. The time intervals for which the rates were determined are indicated on the curves.

TABLE II

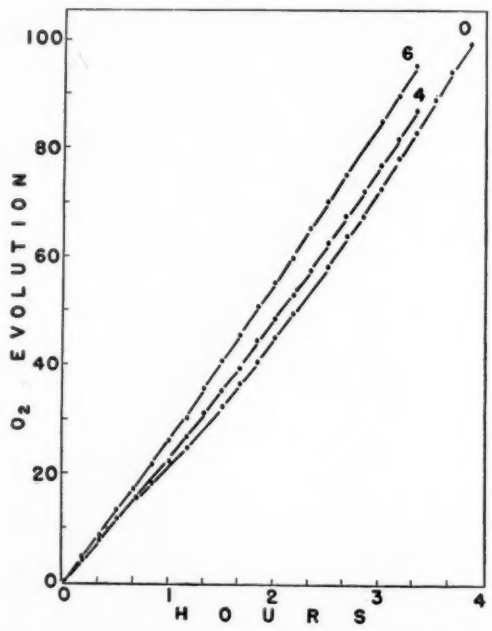
OXYGEN EVOLUTION (1ST HR OF OBSERVATIONS)

LIGHT INTENSITY, ft-c	ABSOLUTE RATES, cmm O_2/cmm CELLS/hr			RATES, % OF CORRESPONDING RATES FOR 0-hr CELLS			RATES, % OF CORRESPONDING RATES AT 200 ft-c		
	AGE OF CELLS, hr								
	0	4	6	0	4	6	0	4	6
200	21	22	25	100	105	119	100	100	100
1,500	58	112	110	100	193	190	276	509	440
2,000	63	127	113	100	202	179	300	577	452

about 130 volumes of oxygen per volume of cells per hour. For the 2nd hour of observation the maximum rate and, possibly, the saturating light intensity were slightly lower than for the 1st hour. For the 3rd hour the maximum rate at light saturation decreased to about 117 volumes of oxygen per volume of cells per hour and saturating light intensity declined to about 1,500 ft-c.

To the effects of the time factor and of light intensity on the photosynthetic gas exchange are added those of the developmental status of cells. In figures 3, 4, and 5 the time course of photosynthesis for 0-, 4-, and 6-hour cells is described at the light intensities of approximately 200 (fig 3), 1,500 (fig 4), and 2,000 (fig 5) ft-c.

The initial rates, those measured during the 1st hour of observations, are given in table II. At a given light intensity the photosynthetic activity increased with the progress in cell development. The increase was small at low light intensity. At 200 ft-c the rate of gas production for 4-hour cells constituted 105 and that for 6-hour cells constituted 119 % of the rate for the 0-hour cells. A further, though small, increase in photosynthetic rate at this light intensity is expected to continue somewhat beyond the 6-hour developmental stage. At higher light intensities the increase in photosynthetic activity with the age of the cells was much greater. At 1,500 and 2,000 ft-c the rates for 4-hour cells constituted, respectively, 193 and 202 % of the corresponding rates for the 0-hour cells. With further progress in cell development, photosynthetic activity began to decline. For 6-hour cells the rates at 1,500 and 2,000 ft-c constituted, respectively, 190 and 179 % of the rates for



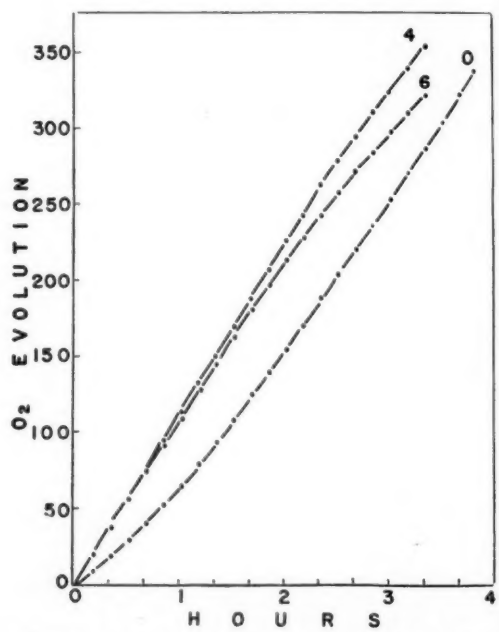


FIG. 3 (*left*). Time course of oxygen evolution at 200 foot-candles in 0-, 4-, and 6-hour cells of Chlorella 7–11–05. Oxygen evolution (apparent photosynthesis) in volumes of gas per packed volume of cells. The developmental stages of cells are indicated in hours on the curves.

FIG. 4 (*right*). Same as figure 3, but at 1,500 ft-c.

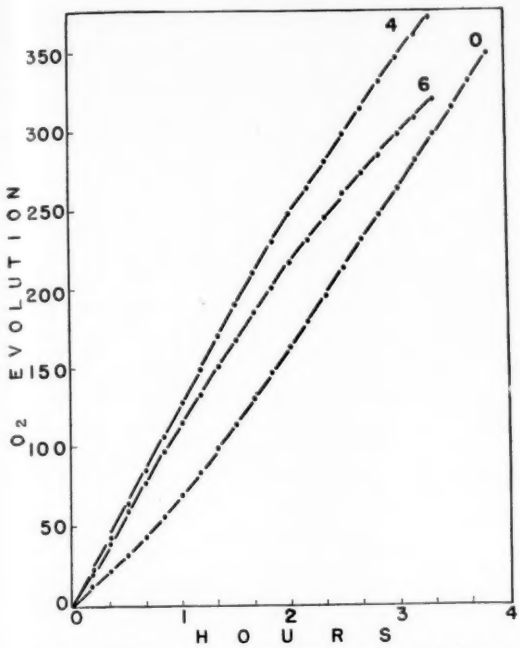


FIG. 5. Time course of oxygen evolution at 2000 ft-c in 0-, 4-, and 6-hour cells of Chlorella 7–11–05. Oxygen evolution (apparent photosynthesis) in volumes of gas per packed volume of cells. The developmental stages of cells are indicated in hours on the curves.

the 0-hour cells. In cells of later developmental stages the photosynthetic activity is expected to decrease to a lower level.

An examination of figures in table II in vertical rows indicates that for a particular kind of cells there was an increase in the initial rate of oxygen evolution with the increase in light intensity. The increase was greatest for 4-hour cells. The initial rate for these cells at 2,000 ft-c constituted 577 % of that at 200 ft-c. For the same span of light intensities the rate for 0-hour cells increased to 300 % and that for 6-hour cells to 452 % of the rate at 200 ft-c. The light intensity of 2,000 ft-c is close to light saturation of photosynthesis for 4-hour cells (figs 1 & 2) and is above light saturation for 0- and 6-hour cells (16). For cells of developmental stages between 0- and 4-hours the saturating light intensity may lie above 2,000 ft-c (16) and the responsiveness to increase in illuminance is estimated to be greater than that indicated in table II for the 4-hour cells.

The general picture is that of increase in responsiveness to increase in light intensity with progress in cell development up to a certain developmental stage. After this developmental stage is passed the responsiveness to the increase in light intensity declines. Under the conditions of these investigations, cell division in Chlorella 7–11–05 started, first in a few cells, after about nine to ten hours of continuous illumination. However, the decline in photosynthetic activity of cells and in their responsiveness to increase in light intensity evidently continued beyond this moment since many cells delayed their divisions to a much later time. With increase in light intensity beyond 2,000 ft-c the maximum photosynthetic activity is expected to coincide with younger cells and to decrease more drastically with progess in cell development.

The pattern of variation in photosynthetic gas exchange, as affected by the developmental status of cells and by the environmental conditions, changes if measurements of the rates are delayed to a later time in a photosynthetic experiment. Data recorded for the 3rd hour of each experiment are presented in table III. The effect of the developmental status of cells at a given light intensity is seen by following figures of table III along the horizontal lines. The rates change little with the change in the developmental status of cells from 0-hour to 4 hours. This was true also for 6-hour cells at the low light intensity (200 ft-c). At higher light intensities the rates for the 6-hour cells were relatively lower: at 1,500 and 2,000 ft-c they constituted, respectively, 89 and 80 % of the corresponding rates for the 0-hour cells.

For cells in a given developmental stage (vertical rows in table III) the rate of oxygen production increased with increase in light intensity. For 0-hour cells the rates at 1,500 and 2,000 ft-c constituted, respectively, 338 and 355 % of the rate at 200 ft-c. The responsiveness of the 0-hour cells to increase in light intensity at this late time of observation was comparable to that of such cells during the 1st hour

TABLE III

OXYGEN EVOLUTION (3RD HR OF OBSERVATIONS)

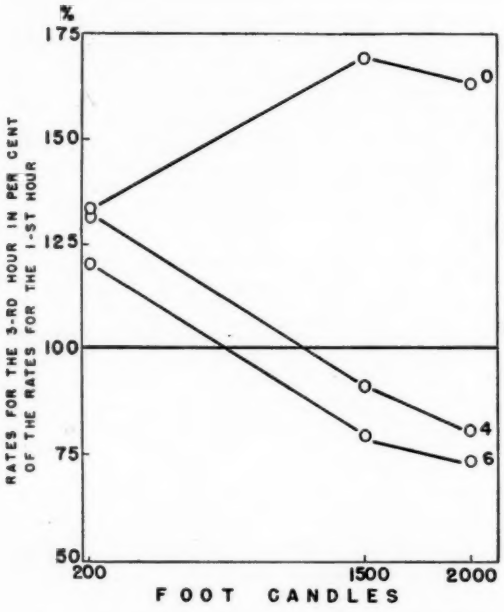
LIGHT INTENSITY, ft-c	ABSOLUTE RATES, cmm O_2/cmm CELLS/hr			RATES, % OF CORRESPONDING RATES FOR 0-hr CELLS			RATES, % OF CORRESPONDING RATES AT 200 ft-c		
	AGE OF CELLS, hr								
	0	4	6	0	4	6	0	4	6
200	29	29	30	100	100	104	100	100	100
1,500	98	102	87	100	104	89	338	352	290
2,000	103	101	82	100	98	80	355	348	273

of observation (table II). For cells in later developmental stages the responsiveness to increased light intensity, as determined during the 3rd hour of observation, was definitely lower than that recorded for the same cells during the 1st hour of observation (table II).

The rates of oxygen evolution during the 3rd hour of observation are now related to those obtained for the 1st hour of measurements. Relative data in figure 6 indicate percentage changes in the rates over 2-hour period for cells in different developmental stages under the influence of a change in light intensity. Comparing rates for the long time intervals makes changes in the rates with time seem less dramatic than they actually are. Rates were changing also within the hourly intervals. If the rates, say, for the last 20 minutes of each experiment were compared with the corresponding rates for the first 20 minutes, then the relative changes would be in many cases more drastic than those indicated in figures 6 and 7. The small number of points indicated in figures 6 and 7 does not permit to connect them with smooth curves though changes in the rates with change in light intensity (fig 6) or with developmental status of cells (fig 7) are, of course, actually gradual.

In 0-hour cells the rates increased in the course of photosynthetic measurements up to 170 % of the initial rates (fig 6). The percentage increase was greatest at some optimal light intensity which probably was somewhat lower than 1,500 ft-c. For older cells the rate of gas production increased in the course of a photosynthetic experiment only at lower light intensity. At 200 ft-c the rates for the 3rd hour of observation constituted for 4-hour cells 130 % and for 6-hour cells 120 % of the corresponding rates for the 1st hour. With increase in light intensity the rate of oxygen evolution can be expected to become fairly constant with time. This probably happens for 4-hour cells at a light intensity close to 1,200 ft-c and for 6-hour cells at one of about 800 to 900 ft-c. With further increase in light intensity, the rates decreased with progress in a photosynthetic experiment. Thus, at 1,500 ft-c the rates of gas evolution for the 3rd hour of observations constituted for the 4-hour cells 90 % and for the 6-hour cells about 80 % of the corresponding rates for the 1st hour of measurements. At 2,000 ft-c the rates for the 3rd hour of observation amounted for the 4- and 6-hour cells only to, respectively, 80 and 73 % of the rates for the 1st hour.

The dependence of change in photosynthetic rates with time at a given light intensity on the developmental status of cells is shown in figure 7. At 200 ft-c the rates increased with time in 0-, 4-, and 6-hour cells. In older cells the increase would probably be smaller. At light intensities of 1,500 and 2,000 ft-c



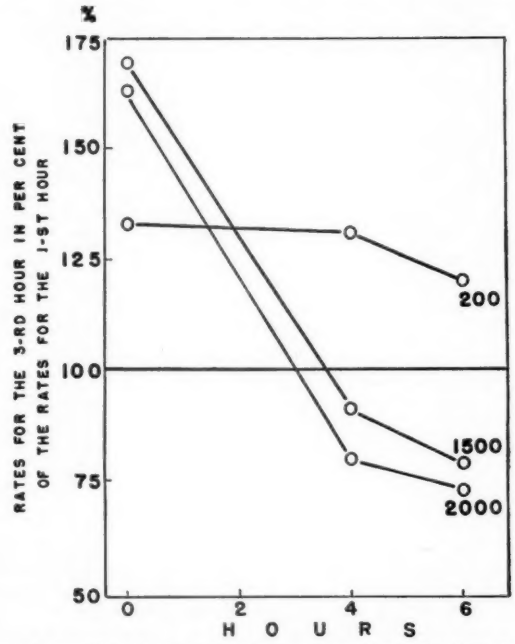


FIG. 6 (*left*). The effect of light intensity (abscissa) on changes in relative rates of oxygen evolution (ordinate) in the course of a photosynthetic experiment in synchronized cultures of Chlorella 7–11–05. The developmental stages of cells are indicated in hours on the curves.

FIG. 7 (*right*). The effect of the age of the cells (abscissa) on changes in relative rates of oxygen evolution (ordinate) in the course of a photosynthetic experiment in synchronized cultures of Chlorella 7–11–05. Light intensities are indicated in foot-candles on the curves.

there was an increase in the rate with time only in younger cells. In older cells there was a decrease in the rate with time, more drastic at more advanced stages in cell development and at greater light intensities. At some intermediate age the rate may be expected to remain fairly constant in the course of a photosynthetic experiment. At 1,500 ft-c the rate will remain constant for at least 3 hours of observation in cells 3½ hours old, and at 2,000 ft-c a steady rate is expected in cells 3 hours old.

The general conclusion is that with both increase in light intensity and progress in cell development the increase in the rates with time diminishes. In a certain range of light intensities and/or at a certain intermediate age the rate may be expected to be more or less unaffected by time. With further increase in light intensity and/or with further progress in cell development, rates decrease with time. An exception to this general rule: rates of oxygen evolution in the youngest cells are affected less and within certain limits are favored by increase in light intensity.

Discussion

Studies on metabolic activity in the course of cell development indicated that concomitant to the cycles of morphological changes there are cyclic fluctuations in metabolic activity, each metabolic cycle corresponding to the cycle of the development of one generation of cells (1, 5, 7, 11, 18, 19, 20). Generally, rates of respiration and photosynthesis decrease toward the end of the life cycle and are the lowest during and/or before daughter cells are liberated from the mother cell wall. With the dark: light synchronization technique metabolic activity in cells of unicellular algae is usually restored during the dark period and reaches its highest level at the beginning of the following light period or after a period of illumination in cells of more advanced developmental stages.

In all those studies metabolic activity has been measured with a manometric technique for intervals of time following harvesting cells from the growth chamber and transferring reaction vessels into illuminated and thermostated water bath. Subsequent studies indicated that, if cells of a predetermined developmental stage were left in manometric flasks for longer periods of time, the time course of photosynthetic gas exchange during continuous 2, 3, or 4 hours of observation had remarkable differences in cells of the successive developmental stages (13, 14, 15, 17).

In younger cells the rate of photosynthetic gas exchange, in 0.02 M potassium dihydrogen phosphate as a suspending fluid (pH 4.5), was gradually increasing during 2 or 3 hours of observation. Under the same experimental conditions photosynthetic activity in cells of advanced developmental stages was decreasing with the time of a manometric experiment. In cells of intermediate developmental stages the activity decreased during the first part of the experiment but later recovered to a level approaching or even surpassing the initial rate at the beginning of the experiment.

Present studies of the phenomenon in carbonate-bicarbonate buffer no. 9 as a suspending fluid essentially substantiated previous observations in acid medium. Due to several advantages of carbonate-bicarbonate buffers they have been extensively used in photosynthetic studies. A stabilizing effect which such buffers exert on carbon dioxide concentration of the suspending fluid was thought to be of importance during intense photosynthesis (9). The feasibility of measuring photosynthetic rates in terms of oxygen evolution by direct method also has been considered of great convenience. The possibility of expressing kinetic data in terms of oxygen, disregarding the exchange in carbon dioxide, is of importance particularly if rates of carbon dioxide exchange undergo considerable changes with time (2, 3). In cells of certain developmental stages synchronized by light-dark technique the rates of both oxygen and carbon dioxide exchange may undergo drastic changes with time (13). Under these conditions no assumed assimilatory quotient is reliable enough for computing results in terms of a particular gas if the work is done by indirect method with one-vessel technique. The indirect two-vessel method is rather inconvenient in studies of the effects of several light intensities, and the results are not likely to be of high precision.

Possible further advantages of carbonate-bicarbonate buffers would be an allegedly better reproducibility of the rates of gas exchange (3) and presumably higher stability of the rates in the course of one photosynthetic experiment. Better reproducibility of the rates is generally accompanied by lower photosynthetic activity and by lower quantum efficiency of cells in carbonate-bicarbonate buffers (3, 4, 10, 21, 22, 23). Better reproducibility of the rates obtained for non-synchronized populations in carbonate-bicarbonate buffers, may not be worth the price of reduced photosynthetic activity and may be misleading. Cellular characteristics of microbial populations are determined on the basis of statistical averages for large numbers of individual cells, which in non-synchronized cultures are in different stages of development. Depending on the developmental status of cells, photosynthetic activity in carbonate-bicarbonate buffers may vary considerably (table II). Thus, reproducibility of results for non-synchronized populations actually depends on skill or luck in obtaining cells of the same age composition in successive experiments.

The stability of the rate of gas exchange with time in carbonate-bicarbonate buffers has been shown to depend in non-synchronized cultures on the previous history of cells (6), on the composition of the buffers (8), on the washing of cells in the process of preparing them for photosynthetic measurements (23), and on light intensity during observations (6). As shown in this report the effects of all these factors are superimposed on the basic differences in developmental status of individual cells. On these differences depends the performance of a cell under a given set of

conditions, the direction and the velocity of change in the rates of metabolism with time, and the responsiveness of the cell to changes in the environmental conditions during observations and to factors that operated during the previous history of cells.

In younger cells the rate generally increases with time at light intensities up to and probably somewhat above 2,000 ft-candles. At a reduced light intensity the rate may increase with time also in older cells. The increase in the rate is probably due to a reaction similar to the upward reaction suggested by observations in an acid medium (13, 14). In older cells the rate of photosynthetic gas exchange usually decreases with time. The decrease in photosynthetic activity is taken as an indication of a downward reaction. This reaction may be similar to the one presumably taking place in acid medium (15) in that in both media the rate of the reaction increases with the progress in cell development and with the increase in light intensity.

A recovery of the rate of gas exchange in the course of a photosynthetic experiment, as had been previously recorded in phosphate buffer (13, 14, 15, 17), was generally not observed in carbonate-bicarbonate buffer at 39° C with a possible exception of the 0-hour cells at 215 ft-c. In these cells the photosynthetic rate dropped after 60 minutes of observation to 80 % and later increased to 130 % of the initial level. The amplitude of fluctuations is small compared to one observed in phosphate buffer (13, 14, 15, 17). An absence or a weak expression of the phenomenon could be attributed to a high pH or lack of phosphorus from the carbonate-bicarbonate buffer. Basically it is probably due to the sluggishness of the upward and the downward reactions in the carbonate-bicarbonate buffer. The initial rate of gas exchange in the carbonate-bicarbonate buffer is lower and the changes in the rate with time are less dramatic than in the phosphate buffer. In this regard carbonate-bicarbonate buffers exert some stabilizing effect on the rate of photosynthetic gas exchange.

It is probable that both the upward reaction and the downward reaction take place in carbonate-bicarbonate buffers in a wide range of external conditions and in cells of diverse developmental stages. The observed change in the rate of gas exchange with time is an outcome of competition between these two reactions. The increase in the rate with time indicates preponderance of the upward reaction. The decrease in the rate indicates prevalence of the downward reaction. In cells of intermediate age these reactions may under certain conditions balance each other and the rate of gas exchange may be constant with time. This, however, should not be taken as indication of the absence of the upward and the downward reactions, but rather of the workings of both of them.

It has been suggested (15) that the upward trend in photosynthetic activity, as observed in time course measurements, might be an expression of the increase in the size of the active part of the photosynthetic mechanism. In younger cells the constructive processes involved can evidently proceed in a wide range of light intensities and in such diverse media as potassium dihydrogen phosphate and carbonate-bicarbonate buffer no. 9. In the absence of external nitrogen the building up of photosynthetic machinery must depend on the amino acid pool as an immediate source of internal nitrogen. An observation that this constructive capacity diminishes with the age of cells corresponds to general understanding that anabolic activity declines with age.

The downward trend in photosynthetic activity with time of an experiment has been taken as an indication of pulling down the photosynthetic machinery (15). Though highly dependent on external conditions this trend is inevitably bound with the progress in cell development. An increase in the rate of this process in cells of advanced developmental stages fits thus a generally accepted idea of the increase in the rate of catabolic processes with age.

Summary

Oxygen evolution in synchronized cultures of Chlorella 7–11–05 was studied at several light intensities in Warburg's carbonate-bicarbonate buffer no. 9 as a suspending fluid. Measurements of photosynthetic activity by direct method in carbonate-bicarbonate buffer essentially substantiated previous observations made at acid pH in phosphate buffer by indirect method.

The initial rate and the time course of the gas exchange during photosynthetic experiments were found to vary with the developmental status of cells and, for cells in a given developmental stage, with light intensity. Generally, in younger cells the rate increased and in older cells it decreased with time. Both the upward and the downward trends for cells of a given age were enhanced by increasing light intensities. At a given light intensity the downward trend was intensified by progress in cell development. As a consequence, the increase in the rate in younger cells diminished with time and the decrease in the rate in older cells was intensified with time.

The conclusion was drawn that the observed rate of gas exchange and its changes with time are the net results of a competition between the upward reaction and the downward reaction. The dependence of these reactions on the processes of building up and pulling down photosynthetic machinery and the connection of the rates of these processes with the anabolic and catabolic activity in the course of cell development was discussed.

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PTERIDINES IN BLUE GREEN ALGAE[1,2]

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The presence of a remarkably high concentration of pteridines in the blue green alga, *Anacystis nidulans*, was demonstrated by Forrest, van Baalen, and Myers in 1957 (2). A number of different pteridines were then isolated and identified. These included 2-amino-4-hydroxypteridine, 2,6-diamino-4-hydroxypteridine (10), a yellow pteridine assigned the structure 2-amino-4-hydroxy-6-propionyl-5,8 or 7,8-dihydropteridine (4), and a glucoside of 2-amino-4-hydroxy-6-(dihydroxypropyl)pteridine (biopterin) (fig 1) (3). Subsequent work has shown that probably all of these compounds are artifacts of isolation. This is based on the fact that Anacystis can be treated in a number of different ways to give varying amounts of these compounds. Thus if the algal cells are simply placed on the origin of a paper chromatogram, and the chromatogram is developed with an ammoniacal solvent, the pteridines which can be identified after development include 2-amino-4-hydroxypteridine, 2,6-diamino-4-hydroxypteridine, and the yellow pteridine accompanied by a light decomposition product. On the other hand, when cells are allowed to stand in the growth medium at 4° for 24 to 48 hours, the principal compound released in the medium is the yellow pteridine. Finally if the cells are acidified with acetic acid and then treated with solid manganese dioxide, the sole pteridine obtained from Anacystis is the glucoside of biopterin (Compound C) (6). These experiments, along with the chemical evidence on the variety of products arising during the reoxidation of a reduced pteridine ring (10) lead to the concept that there is a single compound present in the intact cells which readily breaks down, when the cells are manipulated, to give the products indicated above. This compound probably contains the carbon skeleton of the glucoside of biopterin, but it may be a tetrahydro or dihydro derivative with possibly an exocyclic double bond. Attempts to delineate its structure more rigorously have not been successful.

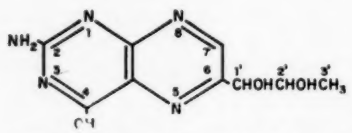

FIG. 1. Structural formula for biopterin. In the glucoside (compound C), the glucose is attached either at the 1′ or at the 2′ carbon.

In preliminary experiments, it was noted that other blue-green algae behaved somewhat similarly to Anacystis when treated under the conditions mentioned above. However, when the acetic acid/manganese dioxide technique, which gives only one pteridine, was discovered and applied to these other algae, it was found that they gave different but closely related blue fluorescent pteridines. A systematic study then revealed that although the compounds produced by this treatment could readily be distinguished, the only difference between them (except in one case to be discussed later) lay in the sugar which was attached to a common molecule, 2-amino-4-hydroxy-6-dihydroxypropylpteridine (biopterin). This was shown by hydrolyzing the compounds with acid after which biopterin could be identified by comparison with an authentic specimen. The acid hydrolysate in any particular case also contained a sugar, which has been identified or tentatively identified by paper chromatography. Since only small quantities of these were available, it has not been possible to apply more rigorous methods of identification to the glycoside components of the molecule.

In the case of one alga, a Synechocystis, acid hydrolysis of the product obtained in the usual way, produced a sugar (glucose) and a pteridine, which was identified by comparison with an authentic sample (5) as 2-amino-4-hydroxy-6-hydroxymethylpteridine. So far as we are aware, this compound has not been previously isolated from natural sources.

MATERIALS & METHODS

Nine strains of blue green algae were examined. With the exception of a marine form and one other, these were obtained through the courtesy of Dr. J.

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Myers from cultures maintained in the Laboratory of Algal Physiology, University of Texas. Their original source is also listed: *Anabaena variabilis* (Kützing), obtained from R. C. Hecker as Cylindrospermum; revised identification by E. G. Pringsheim. *Nostoc muscorum* (Kützing), (Gerloff's strain), obtained from G. C. Gerloff. *Anabaena cylindrica* (Lemmermann), obtained from M. B. Allen. *Anacystis nidulans* (Richter) tentatively identified by F. Drouet. TX27 unidentified strain, however, it is a blue green alga containing phycoerythrin. Synechocystis (Gonzalez strain), obtained from Dr. W. A. Kratz. Synechocystis, high temperature strain, tentatively identified by H. D. Cobb. Synechococcus, obtained from M. B. Allen. Synechocystis, obtained as a lyophilized powder by courtesy of Dr. T. S. Bannister from the Photosynthesis Project, University of Illinois. Apparently, a live culture of this alga is no longer available. Nostoc sp. (Kützing), marine form, isolated by one of us (C. V. B.) from Long Island Sound, N. Y.

The algae were grown in large test tubes (3.5 × 29.5 cm) containing 125 ml of Medium C (7) supplemented with extra nitrate. Illumination was provided by two 20 w fluorescent lamps placed on each side of the culture tubes, giving about 500 ft-c on each side at the level of the tubes. Air, enriched with 0.5 % carbon dioxide was bubbled continuously through the medium. *Anacystis nidulans* and Synechocystis, the two high temperature strains, were grown at 37°; the rest were grown at 25° C. The marine species was grown in a similar manner using the ASP–2 medium of Provasoli et al (8).

The harvested cells (ca. 0.5 g dry wt/tube) from 6 tubes were resuspended in water (5 ml), and dilute acetic acid (2.5 N; 10-15 ml) and manganese dioxide powder (15 mg) were added. After thorough mixing, the whole was centrifuged and the supernatant collected. The cells were washed with aqueous acetone (20 %; 10 ml), recentrifuged, and the supernatants combined.

After evaporation to small bulk (2–4 ml), the extract was streaked on filter paper (Whatman no. 17; 46 × 57 cm) which was then irrigated with the solvent, 1–propanol : 1 % ammonium hydroxide (2 : 1). The appropriate band was located by its blue fluorescense in ultraviolet light (max. emission at 360 mμ) and the material was eluted from it using 1 % ammonium hydroxide. It was then rechromatographed on heavy filter paper, using butanol : acetic acid : water (4 : 1 : 1) as solvent. Elution of the blue fluorescent band from the second chromatogram gave a solution which was used for determination of the ultraviolet spectrum of the compound; for further paper chromatography to determine its R_f values and to compare it with known or similarly isolated materials (table I); and for acid hydrolysis. (In order to provide a convenient reference point the R_f values are recorded in relation to the R_f for biopterin which is given the value one.)

Acid Hydrolysis of Products From Algae. In order to remove interfering materials from eluates from paper chromatographic purification, the blue fluorescent compounds were adsorbed from solution by the addition of a small amount of charcoal (Darco G60). The charcoal was thoroughly washed with water and the fluorescent materials were then eluted from it with ethanol : ammonium hydroxide (1 : 1; 10 ml). This eluate was evaporated to small bulk and treated with the appropriate amount of sulfuric acid. The extent of hydrolysis was followed by checking samples at intervals by paper chromatogra-

Table I

Paper Chromatographic & Hydrolysis Data on Compounds from Blue Green Algae

	4 % Sodium citrate	1–Propanol, 1 % NH_3 (2 : 1)	1–Butanol, acetic acid, water (4 : 1 : 1)	1–Butanol, methyl ethyl ketone, NH_3, water (5 : 3 : 1 : 1)	2–Butanol, formic acid, water (8 : 2 : 5)	Acid strength & time for hydrolysis	Sugar (tentative)
Anabaena var.	1.23	0.97	0.67	0.50	0.91	2 N for 3 hr	6–deoxy–d–glucose
Nostoc mus.	1.23	0.97	0.67	0.50	0.91	2 N for 3 hr	6–deoxy–d–glucose
Anabaena cyl.	1.40	0.94	0.45	0.30	0.80	2 N for 2 hr	Galactose
Anacystis nid.	1.23	0.80	0.37	0.42	0.67	2 N for 2 hr	Glucose
TX27	1.35	0.61	0.45	0.09	0.87	5 N for 4 hr	Xylouronic acid
Synechocystis	1.13	0.83	0.48	0.60	0.84	2 N for 2 hr	Xylose or ribose
Synechocystis*	1.23	0.80	0.37	0.42	0.67	2 N for 2 hr	Glucose
Synechococcus	1.13	0.83	0.48	0.60	0.84	2 N for 2 hr	Xylose or ribose
Synechocystis**	1.34	0.30	0.15	0.0	0.61	2 N for 1 hr	Glucose
Nostoc***	1.23	0.97	0.67	0.50	0.91	2 N for 3 hr	6–deoxy–d–glucose
Biopterin	1.00	1.00	1.00	1.00	1.00		

* High temperature form
** Lyophilyzed preparation
*** Marine form

phy. After complete hydrolysis had taken place, the sulfuric acid was removed by precipitation with barium hydroxide, and the resulting neutral solution was treated to separate the products in either of two ways: I. Charcoal was again added until the solution was no longer fluorescent. The charcoal was removed by centrifugation and treated as before to recover the fluorescent material. This was then compared spectrophotometrically and chromatographically with authentic specimens. The supernatant was evaporated to small bulk and this solution was used for paper chromatographic comparison with known sugars, using butanol : acetic acid : water (4 : 1 : 1) as solvent. II. The hydrolysis mixture was passed through a small column (1 × 3 cm) of Filtrol, previously washed with dilute acetic acid. The fluorescent material was adsorbed at the top of the column. The eluate and water washings were collected, evaporated, and the concentrated solution used for chromatographic comparison with sugars as before. The fluorescent material was washed from the column with 30 % aqueous acetone. Again, the concentrated eluate was used for chromatography, and for ultraviolet absorption spectrophotometry.

Results

Paper chromatography of the algal extracts showed that essentially they contained only one blue fluorescent compound when the cells were treated with acetic acid and manganese dioxide. However, in some cases, several minor, very faint fluorescent bands could be detected. These were not further investigated. The ultraviolet absorption spectra of the various, purified compounds and biopterin were identical with respect to positions of maxima and minima. On hydrolysis, they gave biopterin identified by its ultraviolet absorption spectrum and by paper chromatographic comparison with an authentic specimen. The conditions for hydrolysis and the tentative identification of the sugars thus produced are listed in table I. Several of the compounds yielded biopterin and glucose. The original compounds were then co-chromatographed and shown to be identical. The compound isolated from TX27 was acidic (paper electrophoresis) but, on hydrolysis, it gave biopterin and the acidic portion of the molecule thus resided in the glycosidic moiety. This reacted with the spray reagent to give the characteristic red color of a pentose. It has therefore been tentatively identified as a xylouronic acid.

The one exception to the above statements was provided from the study of the compound obtained from the lyophilized sample of Synechocystis. On acid hydrolysis, as with the other compounds, the ultraviolet absorption spectrum of the hydrolysate was unchanged. However the compound responsible for this ultraviolet absorption spectrum was shown by paper chromatography in a number of solvents to be 2-amino-4-hydroxy-6-hydroxymethylpteridine (table II).

Table II

R_f Values of Pteridine Products After Hydrolysis

	Synechocystis*	Other algae	-6-CH_2OH	Biopterin
4 % Sodium citrate	0.40	0.63	0.40	0.63
1-Propanol, 1 % NH_3 (2 : 1)	0.55	0.56	0.55	0.56
1-Butanol, acetic acid, water (4 : 1 : 1)	0.42	0.33	0.42	0.33
1-Butanol, methyl ethyl ketone, NH_3, water (5 : 3 : 1 : 1)	0.14	0.12	0.14	0.12
2-Butanol, formic acid, water (8 : 2 : 5)	0.64	0.58	0.64	0.58

* Lyophilyzed

Discussion

Since the function of biopterin glucoside (or the natural glucoside from which it is derived) in Anacystis is unknown, it is difficult to assess the biochemical implications of the discovery that one of a number of different sugars may be attached to biopterin in different blue-green algae. The distinct compounds which result may prove to be useful in taxonomic studies within this phylum, since they may provide a simple means for demonstrating a distinction or a similarity between species. A much more extensive study than the one reported herein would have to be made to test the validity of this approach.

Perhaps the most interesting compound described in this paper is the glucoside of 2-amino-4-hydroxy-6-hydroxymethylpteridine isolated from a Synechocystis. 2-Amino-4-hydroxy-6-hydroxymethylpteridine has been implicated, in the form of its tetrahydro derivative, in the biosynthesis of folic acid-like compounds (1, 9).

It is tempting to speculate that the compound isolated from Synechocystis represents an oxidation product from an intermediate in folic acid biosynthesis in this organism, possibly accumulating due to some metabolic disharmony. The other compounds described in this paper would then perform a like function in the specific algae from which they have been isolated. From a theoretical point of view, a similar function for a 6-hydroxymethyl compound and a 6-dihydroxypropyl compound is not at all improbable, since it has been shown that in the reduced form, the side chain in the 6-position of the pteridine ring is

quite labile (10). The three carbon dihydroxypropyl side chain could therefore be replaced by a one carbon fragment, the new molecule then condensing with *p*–aminobenzoylglutamic acid according to the scheme proposed by both Shiota and Brown. In fact, the important intermediate in folic acid biosynthesis may well be 2–amino–4–hydroxy–5,6,7,8–tetrahydropteridine (or a dihydro compound), since it has been shown that this compound, with formaldehyde and *p*–aminobenzoylglutamic acid will condense in the test tube in low yield to give folic acid (unpub. data). Thus the compounds described in this paper would represent end-products derived from compounds capable of supplying the organism with its requirement of reduced 2–amino–4–hydroxypteridine for folic acid biosynthesis. Unfortunately, the available evidence from bacteria is that biopterin and folic acid are not interconvertible (11). However, it would seem worthwhile to repeat such interconversion experiments using blue green algae instead of bacteria.

Summary

A number of new, related compounds have been isolated from a variety of species of blue green algae, using a standard technique. With one exception, these compounds have been shown to be glycosides of biopterin (2–amino–4–hydroxy–6–[1′,2′–dihydroxypropyl] pteridine), differing in the nature of the sugar attached to the side chain. The glucoside of 2–amino–4–hydroxy–6–hydroxymethylpteridine has been isolated from one species. From the method of isolation and other evidence, it seems likely that all of these compounds exist in the intact cell as tetrahydro or dihydro derivatives. The possible significance of these compounds is discussed.

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EFFECT OF BORON ON ELONGATION OF TOMATO ROOT TIPS [1, 2]

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The effect of boron deficiency upon the growing points of both the roots and shoots of plants has been noted many times (2, 10, 11, 12, 18, 21). Several investigators have reported a marked stunting of roots and low root-shoot ratios when boron is deficient for plants growing in sand or solution culture (3, 14). Haynes and Robbins (9) showed that boron, along with calcium, is a necessary component of the root environment for the growth of roots.

Conflicting conclusions are reached on the effect of boron on cellular processes as determined by an analysis of root elongation measurements. Odhnoff (14) concluded that the primary influence of boron was exerted on the cell elongation stage of cell development. Torssell (20) found that arylboric acids promoted root growth very strongly and also concluded that cell elongation was effected. Whittington (25) reported that elongation of field bean seedling radicles, approximately two centimeters long, grown in a complete nutrient solution was 18.5, 13.2, and 12.3 mm per 24 hours for three consecutive days. The elongation of similar radicles in the absence of boron was 10.7, 2.8, and 0 mm per 24 hours. From observations on mitotic stages he concluded that boron deficiency caused a rapid cessation of cell division. Later Whittington (26) suggested that in the absence of boron cell division ceases because abnormalities in the formation of the cell wall prevent the cell from becoming organized for mitosis. Other reports have also indicated a possible relationship between cell division and boron (7).

This paper describes the effects of withholding boron and of low concentrations of boron on the elongation of the tips of roots of intact tomato plants and of excised roots grown in sterile cultures. Relationships between light intensity and root elongation will also be described.

MATERIALS & METHODS

PLANT CULTURE: Tomato plants (*Lycopersicum esculentum* Mill., var. Rutgers) used in these experiments had a high degree of genetic uniformity because they were grown from seed obtained from a seed selection program starting originally from the fruit of one plant. The seeds were germinated in washed quartz sand contained in flats. The seedlings received a complete nutrient solution until they were transplanted. When the seedlings were 2 to 5 cm tall, with the first true leaf expanded and in some instances the second true leaf present, they were washed free of sand and were transplanted to 1 gallon glass jars containing a complete nutrient solution. Each jar held one plant. Aeration of the solutions was started immediately.

A modified Shive's nutrient solution prepared according to the general method of Shive and Robbins (17) was the standard inorganic nutrient solution used in these experiments. The composition was as follows: 0.001 M KH_2PO_4, 0.002 M $MgSO_4$, 0.005 M $Ca(NO_3)_2$, 0.002 M K_2SO_4, 0.0005 M $(NH_4)_2SO_4$, 0.1 ppm B as H_3BO_3, 0.5 ppm Fe as $FeSO_4$, 0.1 ppm Mn as $MnCl_2$, 0.01 ppm Cu as $CuSO_4$, 0.01 ppm Mo as Na_2MoO_4, 0.1 ppm Zn as $ZnSO_4$, and 0.13 ppm Cl as $MnCl_2$. A low nitrogen solution containing 0.0025 M $Ca(NO_3)_2$, 0.00025 M $(NH_4)_2SO_4$, and 0.002 M $CaCl_2$, was substituted during the winter months. Soft glass containers were used for the preparation of solutions and for the growth of the plants. The nutrient solutions were routinely changed every 5 to 7 days with a renewal 1 to 2 days prior to periods of measurement. Lateral buds were removed so that all top growth took place from the apical meristem. Height measurements of the plants were made from the cotyledons to the apical meristems.

Plants used in split root system experiments were supported in specially constructed wooden covers that allowed approximately equal portions of the root system to be placed in adjacent containers. A complete nutrient solution was supplied to both portions of the root system. When the plants were 4 to 6 cm tall the roots were marked and each portion was placed in either a complete or a minus-boron nutrient solution.

ROOT TIP ELONGATION MEASUREMENTS: Root tip elongation was determined by placing India ink marks 5 and 10 mm from the tip and then measuring the subsequent increase in the distance from the tip to the marks. Prior to marking, the root system was rinsed twice in distilled water and then was placed on a moist paper towel for the application of the ink

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marks by means of a chicken feather cut to a fine point. After marking, the plant was placed in a fresh nutrient solution containing the appropriate boron concentration. At the selected time interval of treatment the roots were measured and either returned to the solution for later measurements or were discarded. The measurements of individual roots were made to the nearest 0.5 mm and from 10 to 20 root tips were marked per plant. Selection of plants with roots of approximately equal lengths at the time of marking was necessary for good reproducibility of elongation rates. The close agreement between the average elongation rates of replicate plants (table II) shows the uniformity of results that can be obtained by the root marking technique when applied to carefully selected plants.

Environmental Conditions: The 24 hour experimental period in which root tip elongation was measured began between 8 and 11 AM on the 1st day and concluded on the 2nd day. Mean air temperatures (max + min/2) were calculated for an average experimental 24 hour period beginning at 10:00 AM on the 1st day and concluding on the 2nd day.

Solar radiation was recorded as time-intensity, or solar radiation energy units per day by an Eppley pyroheliometer and a Leeds and Northrup Micromax recording potentiometer with an integrating attachment. Approximately nine radiation units as recorded are equal to 1 gram-calorie per square cm. The average daily value during June 1958, was 6,500 units, whereas, during January 1958, it was 1,824 units. About 50 % of the solar radiation recorded is in the visible portion of the electromagnetic spectrum. Plants growing in the unshaded greenhouse received about 60 to 65 % of the total outside radiation. This was reduced to about 30 to 35 % of the outside radiation when lime was used to shade the greenhouse.

Excised Root Culture: Excised root tips 10 to 12 mm long, used for in vitro root cultures, were obtained from sterile tomato seedlings. The excised root tips were placed, one tip per flask, in 40 ml of a modified White's sterile nutrient solution (13) contained in boron-free glass flasks. A total of 22 cultures was used, half containing 0.1 ppm and half 0 ppm of added boron in the nutrient solution. After growing in the dark at room temperature for 17 days, the roots were removed and measured and observations of their condition were noted.

Results & Discussion

Effect of Boron on Root Tip Elongation of Intact Plants: As will be developed during the presentation of data and in the course of the discussion, withholding boron from the nutrient supply always resulted in an inhibition of root elongation within 24 hours. This clearly implicates boron as influencing cellular processes in an early stage of cell formation or development. The variations in rates of root tip elongation during the 8 month experimental period will be discussed separately.

Table I

Root Tip Elongation 3, 6, 12, & 24 Hours After Withholding Boron*

Boron conc ppm	Average elongation**			
	3 hr mm	6 hr mm	12 hr mm	24 hr mm
0	2.3	4.9	5.1	5.1
0.1	2.3	7.1	16.2	31.5

* March 17, 1958.
** Average elongation of 10 to 20 tips on one plant, with different plants being measured at each time interval.

The results of an experiment to determine how soon root tip elongation stopped after the boron was withheld from the nutrient solution are given in table I. To avoid repeated handling of the roots, each measurement was obtained from a different plant for each level of boron at each time period. Elongation may stop as early as 6 hours after the boron is withheld from the nutrient solution. The rapid stopping of elongation soon after boron is withheld demonstrates that the boron previously taken in by the plant is not readily translocated to the root tip. It also suggests that the growing root tip does not accumulate any sizable amount of boron which could be used when boron is no longer externally available.

The effect of five different boron concentrations on the elongation of tomato root tips was determined. In this experiment two plants per concentration of boron were used and elongation of root tips of each plant was measured 24 and 72 hours after the roots were marked. The results, presented in table II, show that tomato roots can respond very sensitively to a low concentration of boron in the nutrient solution. It also shows that during the first 24 hours, 0.01 ppm of boron was adequate to sustain the maximum rate of elongation. However, the amount of boron available was no longer adequate to yield the

Table II

Effect of Different Levels of Boron on Elongation* of Root Tips of Tomato Plants

Boron conc ppm	Average elongation**					
	24 hr			72 hr		
	Plant 1 mm	Plant 2 mm	Avg. mm	Plant 1 mm	Plant 2 mm	Avg. mm
0	5.3	4.9	5.1	6.6	6.0	6.3
0.0001	5.7	5.7	5.7	10.9	10.5	10.7
0.001	21.1	20.9	21.0	70.2	73.1	71.7
0.01	34.6	35.9	35.3	99.0	98.9	99.0
0.1	33.6	33.6	33.6	112.1	112.0	112.1

* March 28, 1958.
** Average elongation of 10 to 20 tips.

same amount of elongation over a 72 hour period that was given by 0.1 ppm boron. The difference after 72 hours between average elongation on 0.1 ppm boron (112.1 mm) and on 0.01 ppm boron (99.0 mm) is significant at the 5 % level.

The translocation of boron and its effect on root tip elongation was studied by utilizing the split root system technique. The data in table III show that the root tips of the portion of the root system receiving no boron (3B) did not elongate to any appreciable extent after 1 day in the minus-boron nutrient solution, even though the root tips of the other portion of the root system (3A) were receiving 0.1 ppm boron and were elongating at the normal rate. There was no significant difference (1 % level) between the amount of elongation of root tips of the plant in which both root portions were exposed to a minus-boron nutrient solution (plant 2) and the root portion (B) exposed to a minus-boron nutrient solution of the plant (plant 3) on which the other root portion (A) received 0.1 ppm boron. Several other similar experiments confirmed these results. These experiments demonstrated that boron was not translocated to any appreciable extent from the root portion in a nutrient solution containing 0.1 ppm boron to the other root portion that did not receive any boron. The observations are in agreement with the conclusion of Haynes and Robbins (9) that boron is a necessary component of the external environment of roots.

Symptoms of Boron Deficiency: Visible symptoms of boron deficiency on tomato roots can be seen 24 hours after the plants supplied with adequate boron are placed in a nutrient solution containing 0 ppm boron. The typical symptoms evident are a browning of the root tips and the appearance of lateral roots close to the primary root tip. The terminal two to three mm of the root tip is brown and the lateral roots, which are normally present several centimeters (5–7 cm) behind the tip of the primary root appear within five to seven mm of the root tip.

Another symptom of boron deficiency on tomato roots is the absence of a characteristic white fluorescence that is seen when healthy root tips are illuminated with ultra-violet (360–370 mμ). As the brown color due to boron deficiency develops, the intensity of the fluorescence from this same area decreases. Boron deficient tips which turned brown and lost their fluorescence did not resume elongation when again supplied with boron. In contrast Spurr (19) observed that a fluorescence appeared prior to boron deficiency symptoms on celery petioles.

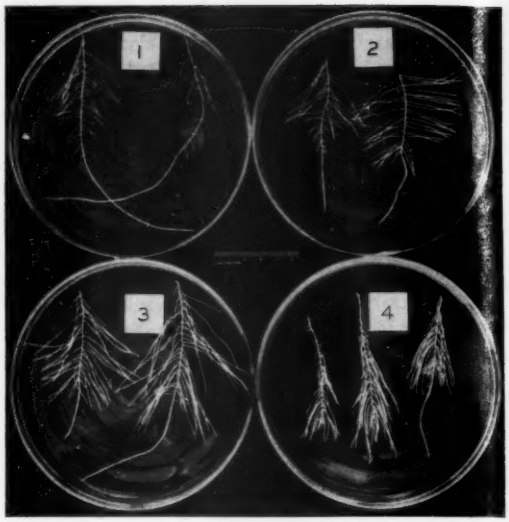

Fig. 1. Roots of tomato plants exposed to a minus-boron nutrient solution followed by recovery in a complete solution. Treatments: 1. Complete, 2. —B for 1 day, 3. —B for 2 days, 4. —B for 3 days. Recovery periods 9, 8, 7, and 6 days, respectively.

Several experiments were performed to determine how long the roots of tomato plants can be exposed to a minus-boron nutrient solution and then resume growth when again supplied with boron. In one of these experiments three plants were exposed to a minus-boron nutrient solution for 1, 2, and 3 days, one plant for each time period. At the end of the exposure periods root tip elongation was measured and each plant was then placed in a complete nutrient solution containing 0.1 ppm of boron. Nine days from the start of the experiment root tip elongation was again measured, thus giving recovery periods of 8, 7, and 6 days in a complete nutrient solution. An identical schedule of measurements was followed for plants that were grown continuously on an adequate (0.1 ppm) level of boron. The root tip elongation measurements for this experiment are given in table IV. Figure 1 shows the pattern of root development at the end of the recovery periods.

The results in table IV demonstrate that exposure to a minus-boron nutrient solution for 1, 2, and 3 days results, in all instances, in a marked decrease in the rate of elongation of the main tips as compared with those of plants grown on a complete nutrient

Table III

Effect of Boron on Elongation* of Tomato Root Tips in Split Root Systems

Plant	Root portion	Boron conc ppm	Average elongation**		
			1 Day mm	2 Days mm	6 Days mm
1	A	0.1	26.1	55.8	157.3
	B	0.1	28.7	56.9	150.8
2	A	0	3.5	3.8	3.7
	B	0	3.4	3.9	4.0
3	A	0.1	26.8	57.6	169.5
	B	0	3.9	4.1	4.4

* April 1, 1958.
** Average elongation of six tips per root portion.

solution. Upon return to a nutrient solution containing 0.1 ppm boron, the rate of elongation of the main root tips did not increase to any appreciable extent.

The recovery of tomato plants from boron deficiency follows a uniform pattern. This recovery pattern is seen on the root growth of plants from which boron was withheld and to which an adequate amount (0.1 ppm) of boron was supplied following the withholding period. Treatment 1 (fig 1) demonstrates that the first lateral roots on the plants grown with a continuous supply of boron are seen at a considerable distance from the root apex. It is noted that the laterals are rather short and that dominance of growth by elongation is maintained by the main root tip. Although elongation of the main root was resumed slowly when boron was again made available to the boron-deficient plants, the laterals also elongated. It appears that, associated with the inhibition of elongation of the main root tip, the dominance of the main tip is lost. In treatment 4, minus-boron for 3 days, it was noted that the laterals, in the majority of all the roots observed, were closer to the root tip than for treatments 2 or 3, and that several of the main root tips appeared to be dead.

During recovery of tomato roots from boron deficiency new roots were initiated at varying distances behind the apical meristems. Even the small laterals that formed during the deficiency period, near the apex of a terminal root, sometimes developed sublaterals. For example, one small lateral root 3 mm long, that developed during the development of boron deficiency, was located approximately seven millimeters behind the terminal apex, had three sublaterals on its terminal 2 mm. Also, new roots developed very close to the main terminal meristem, within 1 to 3 mm from the apex. This indicates that, although root elongation stops in the absence of boron, maturation, evidenced by the initiation of lateral roots, progresses down the root. Lateral roots emerging close to the injured tip were coming from an area that was brownish in color, and therefore apparently not all of the cells in these brown colored tips were dead. However, root tips that are brown do not elongate when supplied with boron. New laterals formed in the absence of boron continue to elongate if they are supplied with boron before they turn brown.

Odhnoff (14) reported on the growth of the roots of bean plants supplied intermittently with boron in the nutrient solution every five days. When boron became deficient after 5 days the main tips died and the laterals began first to elongate, but then stopped. On the addition of boron other laterals resumed elongation. This treatment was continued several times and caused a bushy appearance of the boron deficient roots. This bushy appearance was also observed in the present investigation on tomato roots after boron was withheld and again supplied to the nutrient solution in which the plants were growing (fig 1).

Sommer and Sorokin (18) reported that a new, long, slender root was observed at the end of an enlarged apex within 48 hours after boron was supplied to pea plants showing an extreme degree of injury from lack of boron in the nutrient solution. They did not mention any observations on the pattern of the growth of the lateral roots after boron was added to the solutions from which the boron was withheld. In the present investigation a similar swelling or enlargement was also observed on all of the tomato roots that recovered from exposure to a minus-boron nutrient solution (see the right hand root of treatment 2 in fig 1).

Limited observations on the internal symptoms of boron deficiency in tomato root tips were also obtained in the present investigation. These symptoms appeared rapidly (24 hr) after boron was withheld from the nutrient solution. The first such symptoms noted were in the inner portion of the cortex in the post-meristematic region of the root tip. The cortex was affected earlier than the vascular cylinder and the meristem appeared least affected. The first symptom of boron deficiency appeared as a darker staining of the cytoplasm and this was followed by a disintegration of the cytoplasm, resulting in empty, apparently dead cells. The cell walls of some, but not all, of the dead cells appeared thicker than the cell walls of cells containing cytoplasm. Xylem development and lateral root initiation occurred closer to the apex in boron deficient roots than in roots supplied with adequate boron. Alexander (1) observed similar boron deficiency symptoms in squash roots 2 days after boron was withheld from plants grown in sand culture.

The observations that boron affects the post-meristematic regions of the root and shoot (16, 21) are parallel to the conclusion reached in the present investigation. Although boron affects the meristematic regions of the root and shoot, the first visible internal effects were not in the meristem proper. Also, in roots that had advanced symptoms of boron deficiency the meristem was not nearly as severely affected as were the tissues directly behind it. Severely affected meristems (5, 16, 18, 21, 22) may be found only after a long continued period of boron de-

TABLE IV

ELONGATION OF TIPS OF TOMATO ROOTS EXPOSED TO NUTRIENT SOLUTIONS WITH & WITHOUT BORON* & SUBSEQUENT ELONGATION WHEN PLACED IN COMPLETE NUTRIENT SOLUTIONS

EXPOSURE PERIOD			RECOVERY PERIOD		
DAYS	BORON CONC ppm	ELONGATION** mm/DAY	DAYS	BORON CONC ppm	ELONGATION** mm/DAY
1	0.1	15.6	8	0.1	15.4
2	0.1	19.3	7	0.1	14.8
3	0.1	25.4	6	0.1	16.2
1	0	5.7	8	0.1	4.5
2	0	3.4	7	0.1	6.9
3	0	1.6	6	0.1	4.2

* Experiments started on February 12, 1958.
** Average elongation of 10 to 20 tips on one plant.

ficiency. Whittington (26), however, found the first effects of boron deficiency in the meristematic zone.

From the preceding results and observations, and from the early appearance of internal symptoms of boron deficiency in tomato root tips, it appears that boron definitely influences early stages of cell development. Whether or not boron affects cell division or cell elongation cannot be ascertained from this study. However, these results clearly demonstrate that to detect and study the earliest, and possibly the primary effects of boron deficiency on roots, observations must be started very soon after treatments to induce boron deficiency are initiated. Otherwise, secondary effects of boron deficiency may confuse the interpretation of the observations.

Effect of Boron & Sucrose on Elongation of Excised & Intact Roots: Excised root tips were grown in a modified White's medium in sterile culture to determine the effect of boron on the growth of roots in the absence of the top of the plant. The results in table V demonstrate that without boron in the nutrient medium root tips elongated more slowly than those of roots receiving 0.1 ppm of boron. However, the most interesting observation is that elongation occurred at only a slightly lower rate over a long period of time, i.e., 17 days, in the absence of added boron. No differences in color were apparent between the roots and root tips receiving 0.1 ppm boron and those receiving 0 ppm of added boron. This is in marked contrast to the roots of intact plants exposed to a minus-boron nutrient solution, when root elongation is essentially stopped about 6 hours after the boron was withheld, and the terminal 1 to 3 mm of the tip turns from a normal greenish-yellow color to a distinctive brown color after about 24 hours.

White (24) reported that tomato roots grown in sterile culture continue to grow reasonably well (76 % of the control) after three passages, each 1 week long, through nutrient solutions from which the boron was withheld. Gautheret (8) found that it was necessary to make three passages to obtain death of carrot tissue cultures in the absence of boron. The present results agree with these observations.

Several experiments were performed in an effort to determine the cause of the differences observed between the rates of elongation of roots grown without boron in sterile root culture (table V) and of roots of intact plants grown without boron by regular nutrient solution methods.

The modified White's solution used with non-sterile solution culture techniques and Shive's nutrient solution were compared for their ability to support root elongation with and without sucrose and boron. In some instances, bacterial contamination was evident 24 hours after the start of the experiments in cultures which contained sugars, but it was concluded that such contamination did not significantly affect the validity of the results during the first 24 hours because results of experiments in which bacterial contamination was evident did not differ from those in which contamination was not found. The results of this experiment, given in table VI, show that a complete White's solution inhibits root elongation compared to White's solution from which the sucrose was withheld. In the absence of boron, sucrose did not stimulate root elongation. White's solution without boron gave a slightly greater root elongation than Shive's solution without boron. The root tips were brown, indicating permanent boron deficiency injury, when boron was absent from either White's or Shive's solution.

The nutrients of White's solution that are different from the nutrients in Shive's solution were tested individually and in combination on intact plants by adding them to Shive's solution from which the boron was withheld. None of these components overcame the inhibition of root elongation caused by withholding boron from the nutrient solution. On the basis of these results and from the data in table VI, it is concluded that White's medium does not contain sufficient boron or boron sparing elements to permit normal root elongation of intact plants when boron is withheld from the nutrient medium. Glucose, fructose, and riboflavin were also tested to see whether or not they could overcome the inhibition of root elongation of intact tomato plants in the absence of

Table V

Effect of Boron on Elongation of Tips of Excised Tomato Roots Gbown in Sterile Culture in White's Medium

Boron conc	Elongation*	
	17 Days	Avg./day
ppm	mm	mm
0	161	9.5
0.1	244	14.4

* Average of 11 tips per treatment.

Table VI

Elongation of Root Tips of Intact Tomato Plants Grown in Non-Sterile Shive's & in White's Nutrient Solutions Containing Different Combinations of Sucrose & Boron*

Nutrient solution	Sucrose conc %	Boron conc ppm	Avg. elongation** mm
Shive's	0	0.1	26.5
Shive's	0	0	3.2
White's	2	0.1	14.3
White's	0	0.1	29.0
White's	2	0	5.6
White's	0	0	6.6

* April 25, 1958

** Average elongation in 24 hours of 10 to 20 tips on one plant.

boron. None of these substances had a beneficial effect on the elongation of roots in the absence of boron. The root tips of all plants that did not receive boron were brown in color.

A difference between the physiology of excised and of intact roots is indicated by their responses when boron was withheld from the nutrient medium. This could be true since tomato roots grown in White's medium seldom form a phloem, but they do form a cambium and sometimes a normal xylem (4). Gauch and Dugger (6, 7) presented a theory on boron action based on the concept that "boron deficiency symptoms are an expression of sugar deficiency in the cambia, stem tips, root tips, and flowers or fruits". However, Palser and McIlrath (15) showed that there was no decrease in the percentage of carbohydrate in the roots of cotton under boron deficiency conditions. Odhnoff (14) found an increase in reducing and non-reducing sugars in roots and leaves of boron-deficient bean seedlings compared to normal ones. She suggested that sugar continues to be translocated, but that it accumulates because root growth is inhibited. Whittington (26) also was unable to overcome boron deficiency inhibition of root growth in field beans by supplying sucrose to the nutrient solution. It would appear that sucrose supplied externally to roots would be available for at least temporary alleviation of boron deficiency, if the symptoms were caused by sucrose deficiency. The results of the present investigation do not support the hypothesis that boron deficiency is only a deficiency of sugar in the affected regions of the plant. The next section of this paper presents data which show that various environmental factors which affect the shoot directly also indirectly affect the growth and boron deficiency responses of the roots. It seems unlikely that carbohydrate supply is the sole link between the shoot and these root responses.

Relationships Between Boron, Light Intensity, Root Tip Elongation, & Plant Height: The rate of root tip elongation of plants supplied with 0.1 ppm of boron increased from a minimum rate in November 1957, to a maximum rate in March

Table VII

Average Root Tip Elongation During Each 24 Hour Period of Tomato Plants Grown in Nutrient Solutions*

Date expt. started	Avg. elongation** 0.1 ppm B mm	Avg. elongation** 0 ppm B mm	Avg. ht. cm	Solar radiation 1st Day Units	Solar radiation 2nd Day Units	Mean air temp. ° F
11/25/57	8.8***	...	13.0	1,680	2,695	64
11/29/57	7.7	2.8	15.0	2,088	258	66
1/3/58	8.2	5.2	24.0	2,439	2,677	66
1/9	9.9	6.1	30.0	2,847	2,635	67
2/7	8.4	3.9	9.5	195	2,863	62
2/12	15.6	5.7	10.5	1,592	3,392	67
2/26	17.5	4.2	5.8	2,655	518	66
3/10†	27.1	4.3	5.0	5,009	5,628	70
3/17	31.5	5.1	3.3	4,204	4,506	67
3/24	23.1	5.7	6.3	5,107	1,754	69
3/28	33.6***	5.1***	3.3	4,938	6,798	66
4/1	27.4	3.5	4.5	5,179	7,078	63
4/12	23.6	4.3	5.3	2,257	7,768	65
4/18	25.2	4.9	12.0	6,307	7,688	76
4/23	26.3	5.2	5.8	3,055	5,802	75
4/25	26.5	3.2	7.5	6,983	8,233	71
5/8	21.4	9.4	9.0	3,271	7,627	69
5/19	23.3	3.3	7.0	4,407	4,578	78
6/4	28.0	3.4	6.3	8,273	8,188	70
6/20††	15.6	3.8	6.8	1,510	2,130	65
6/25	24.3	1.9	10.8	7,210	6,121	85

* With and without boron in a series of tests during an 8 month period with plant height, solar radiation, and air temperature.
** Average elongation in 24 hours of 10 to 20 root tips on one plant.
*** Average of two plants.
† Changed from low nitrogen solution.
†† Lime shading applied to greenhouse glass.

1958, which was maintained in a series of tests through June, at the end of the 8 month period. The data for the root tip elongation in each 24 hour experimental period, the date the experiment was started, boron level, solar radiation for the 1st and 2nd experimental days, and mean air temperature for the 24 hour experimental period are given in table VII. To account for the change in rates of root elongation in different months of the year, statistical correlations of root tip elongation rates were made with A. solar radiation of selected days, B. mean air temperature of the average 24 hour experimental period, and C. average height of the plants at the time the experiments were started. Similar correlations were also calculated for the rates of root tip elongation of plants from which the boron was withheld for the 24 hour experimental period. Table VIII lists the correlation coefficients which were calculated for these variables.

The most prominent feature of the values in table VIII for plants supplied with boron is that all correlations of root tip elongation with solar radiation of selected days are positive, whereas there is a significant negative correlation with plant height. The highest correlation between root tip elongation and solar radiation occurred when the radiation values of the 1st and 2nd days were combined.

The correlation of root elongation with solar radiation during the measurement period is presumably higher than that which was found for the combined radiation of the 1st and 2nd days, because some of the radiation came before and some after the measurement period. The lowest correlation of root elongation with solar radiation was found for the day before the experiment was started. A comparison of the correlation of root elongation with the solar radiation of the day before the experiment was started (+0.342), with the combined radiation of the 1st and 2nd days (+0.794), indicates that root elongation is closely related to the prevailing solar radiation.

TABLE VIII

TOTAL CORRELATIONS OF ROOT ELONGATION WITH SOLAR RADIATION, HEIGHT OF PLANTS, & AIR TEMPERATURE FOR TOMATO PLANTS WITH & WITHOUT ADDED BORON IN NUTRIENT SOLUTION

AVG. ROOT ELONGATION IN 24 hr vs:	CORRELATION COEFFICIENT** ppm OF BORON	
	0.1	0
	r	r
1. S.R.* week before	+0.573	−0.410
2. S.R. day before	+0.342	−0.359
3. S.R. 1st day	+0.736	−0.588
4. S.R. 2nd day	+0.720	+0.063
5. S.R. 1st & 2nd day	+0.794	−0.117
6. Plant height	−0.715	+0.027
7. Air temperature	+0.377	−0.246

* S.R. = Solar radiation
** Significance: 5 % level = 0.444. 1 % level = 0.561.

Higher light intensities may result in the production of more carbohydrate which would be available for higher rates of root growth than in the case of lower light intensities. The significant (1 % level) negative correlation between plant height at the beginning of the experiment and root elongation means that the rate of root tip elongation of tall plants was less than that of small plants. This may mean that the smaller number of root tips of the smaller plants were better supplied with carbohydrates (and/or other nutrients) than the relatively larger number of root tips of the larger plants.

Wassink (23) has recently reviewed experiments by himself and Richardson in which they found, for Acer species seedlings, a linear relation between the rate of root growth and light intensity up to about 4,000 lux. Increases of light intensity up to 9,500 lux also gave a small increase (not linear) in rate of root growth. A comparison of root growth with light intensity showed a close similarity with a photosynthesis curve. It was also found that light saturation for root growth was also about the same as for photosynthesis measured for single leaves. It was concluded that in the young Acer seedlings root growth is intimately related to the photosynthesis of the shoot. Although the data reported here are not as extensive as those reviewed by Wassink (23), they nevertheless confirm his conclusions on the closeness of the relationship between light intensity (solar radiation), photosynthesis, and rates of root elongation.

In the absence of boron it was found that root tip elongation was not significantly related either to root tip elongation with 0.1 ppm boron (+0.034) or to plant height (+0.027). The closest relationship between root tip elongation and radiation was found with the radiation of the 1st day (−0.588). This correlation was significant at the 1 % level of probability. Essentially no elongation occurred during the 2nd day portion of the experimental period when boron was withheld, which accounts for the lack of correlation of root growth with solar radiation on the 2nd day.

The results in table VIII also show that under the conditions of these experiments, root tip elongation both in the presence and in the absence of added boron was not significantly correlated with the mean air temperature during the experimental 24 hour period. Unpublished work done in this laboratory by M. Borys, suggests that low temperatures may have a sparing effect on the inhibition of root elongation in the absence of boron.

SUMMARY

A study has been made of the effects of boron deficiency on the elongation of root tips of tomato plants grown in nutrient solution. External symptoms of boron deficiency were detected within 24 hours after boron was withheld from the nutrient solution. These symptoms consisted of the cessation of root elongation as early as 6 hours after boron was withheld, and the

development of a brown color and loss of fluorescence in the terminal portion of the root tip. Brown root tips did not resume elongation when given an adequate supply of boron. However, laterals emerged from the brown area and continued to elongate if boron was again added to the solution before the lateral tips turned brown.

Boron supplied in the nutrient solution to one portion of a split root system did not support elongation of the root tips on the portion of a divided root system that did not receive a supply of boron.

Excised tomato root tips grown in sterile culture in White's medium for 17 days, without an added supply of boron, did not show the same striking inhibition of root elongation that was exhibited by root tips of intact plants 24 hours after the boron was withheld from the nutrient solution. Neither White's medium nor any of its components when added to the standard inorganic nutrient solution relieved the inhibition of root elongation of intact plants caused by the absence of boron from the nutrient solution. Boron deficiency symptoms of roots of intact tomato plants are not relieved by supplying sugars to the roots.

Root elongation of intact tomato plants grown in solution culture with an adequate supply of boron (0.1 ppm) was significantly correlated directly with the total solar radiation of the 1st and 2nd days of the experimental period and inversely with the height of the plants. When boron was withheld from the nutrient solution, elongation was negatively and significantly correlated with the total solar radiation of the 1st day of the experimental period.

The first internal symptoms of boron deficiency in tomato root tips developed within 24 hours after boron was withheld, and were evident in the 1st mm of the post-meristematic region of the tip. The first symptoms appeared to be a disintegration of the protoplasts of some of the cortical cells. It is concluded that root tip elongation stops when boron is not available to the root because boron definitely influences early stages of cell development in tomato root tips.

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LIGHT DEPENDENT OXYGEN METABOLISM OF CHLOROPLAST PREPARATIONS. III. PHOTOOXIDATION OF ASCORBIC ACID[1,2]

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Ascorbic acid is a normal constituent not only of the chloroplasts but also of the other protoplasmic components of the cells of green leaves. In spite of its known widespread occurrence in plants, few functions of ascorbic acid in plant metabolism have been established (see review by Mapson, 15). The participation of ascorbic acid as an intermediate hydrogen carrier in photosynthesis has been suggested (16, 17) and certainly the experiments of Krasnovsky on the photochemical reduction of chlorophyll in vitro by ascorbic acid (13, 14) have added new interest to these suggestions. Ascorbic acid was at one time considered to be a necessary component of the photosynthetic phosphorylation system but more recently it has been regarded as having a protective role in preventing inactivation of essential components of the chloroplasts (Arnon, 1).

Soon after he discovered that chloroplasts could use oxygen as a Hill reagent, Mehler (18, 19) reported that rates of net oxygen uptake in the light by reaction mixtures containing chloroplasts, ethanol, and catalase were stimulated several fold if the chloroplasts had first reduced quinone. To determine if it was necessary for chloroplasts to reduce quinone in the light, Mehler added ascorbic acid to the reaction mixture to reduce quinone in the dark. When an excess of ascorbic acid was added, an extremely rapid oxygen consumption occurred on illumination. The nature of this rapid uptake in the presence of ascorbic acid was of particular interest because of the apparent increase in the efficiency of utilization of light energy.

Later, Vernon and Kamen compared the photooxidations of various redox couples by spinach leaf homogenates and sonic extracts of *Rhodospirillum rubrum* (21). The most active couple which they found was the combination of 2,6-dichlorophenolindophenol (DPIP, acting as an electron carrier) and ascorbic acid (acting as the ultimate electron donor). In both plant and bacterial extracts, oxygen uptake in the light continued until oxygen equivalent to the excess ascorbic acid in the system had been consumed. The nature of this rapid DPIP-mediated photooxidation of ascorbic acid has since been investigated using tracer oxygen techniques (Habermann & Vernon, 10). The results of these studies support the hypothesis that ascorbic acid can be oxidized by the oxidized product of photolysis.

This paper describes investigations of the mechanism of the photooxidation of ascorbic acid mediated by chloroplast reaction systems.[4] Addition of ascorbic acid during either a Mehler or exchange reaction results in an immediate acceleration of the rate of net oxygen uptake. Just as the rates of the Mehler and exchange reactions are accelerated after a chloroplast preparation has reduced quinone (19, 8), previous reduction of quinone has a stimulating effect on rates of subsequent ascorbic acid oxidation by such chloroplast preparations. The use of tracer oxygen (which makes possible the simultaneous measurement of rates of oxygen consumption & production) indicated that increases in rates of net oxygen uptake during the oxidation of ascorbic acid are *not* a consequence of an additional oxygen-consuming reaction being super-

Hill reaction (production only):

$$\begin{aligned} 4H_2O &\longrightarrow 4(H) + 4(OH) \\ 2Q + 4(H) &\longrightarrow 2QH_2 \\ 4(OH) &\longrightarrow 2H_2O + O_2 \\ \hline 2H_2O + 2Q &\longrightarrow 2QH_2 + O_2 \end{aligned}$$

Exchange reaction (no net change in oxygen tension, i.e., production equal to consumption):

$$\begin{aligned} 4H_2O &\longrightarrow 4(H) + 4(OH) \\ 2O_2^* + 4(H) &\longrightarrow 2H_2O_2^* \\ 4(OH) &\longrightarrow 2H_2O + O_2 \\ 2H_2O_2^* &\longrightarrow 2H_2O^* + O_2^* \\ \hline 2H_2O + O_2^* &\longrightarrow O_2 + 2H_2O^* \end{aligned}$$

Mehler reaction (production less than consumption of oxygen):

$$\begin{aligned} 4H_2O &\longrightarrow 4(H) + 4(OH) \\ 2O_2^* + 4(H) &\longrightarrow 2H_2O_2^* \\ 4(OH) &\longrightarrow 2H_2O + O_2 \\ 2H_2^*O_2^* + 2CH_3CH_2OH &\longrightarrow 4H_2O^* + 2CH_3CHO \\ \hline 2H_2O + 2\,CH_3CH_2OH + 2O_2^* &\\ \rightarrow 2\,CH_3CHO + 4H_2O^* + O_2 & \end{aligned}$$

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[2] The contents of this paper are part of a thesis submitted to the graduate faculty of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree.

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[4] These reactions can be defined operationally in terms of oxygen metabolism according to the following equations:

imposed on the normal pattern of oxygen metabolism of these chloroplast reactions, but that ascorbic acid is oxidized by reaction with either the oxidized product of photolysis or an intermediate in the pathway to oxygen production. Preliminary reports of these findings were presented at the A.I.B.S. meetings in East Lansing, Michigan, and at the Gatlinburg conference on photosynthesis (7, 9).

Materials & Methods

Chloroplasts were prepared from mature leaves of *Phytolacca americana* L. (pokeweed) according to procedures previously described (Habermann, 8). All reactions were run in conventional rectangular Warburg vessels. Light from a bank of fluorescent bulbs mounted vertically behind the back window of a constant temperature water bath was reflected onto the bottoms of the experimental vessels by a mirror mounted at a 45° angle. Manometric experiments were run at 18.8° C, tracer experiments at 23° C. Adaptation of the mass spectrometer (CEC Model 21–201) has been described by Brown, Nier, and van Norman (4) and further modifications of the apparatus have been described by Johnston and Brown (11).

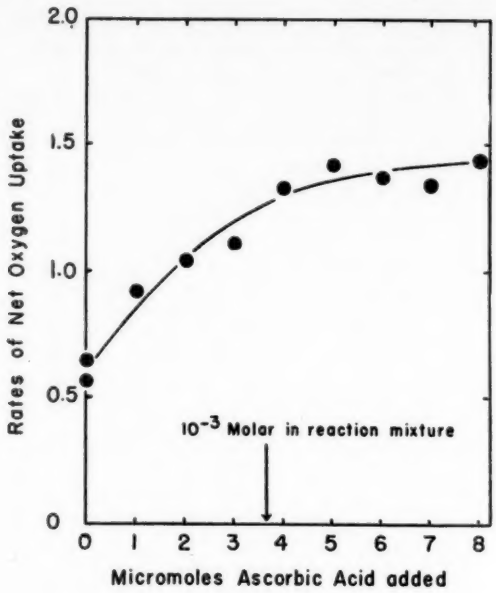
The gas phase for all manometric experiments was air. In experiments using tracer oxygen, the vessel containing 3 cc chloroplast suspensions plus other components of the reaction mixture was attached to the leak assembly of the mass spectrometer and flushed with helium for 5 minutes. Oxygen enriched with mass 34 ($O^{16}O^{18}$) was then added so that the resulting gas phase was composed of 2 to 3 % oxygen in helium. Rates of oxygen consumption and production were calculated from concentrations of mass 32 (ordinary oxygen) and mass 34 which were recorded automatically. Methods of calculation and considerations of possible corrections were discussed in a recent paper by Brown and Weis (5).

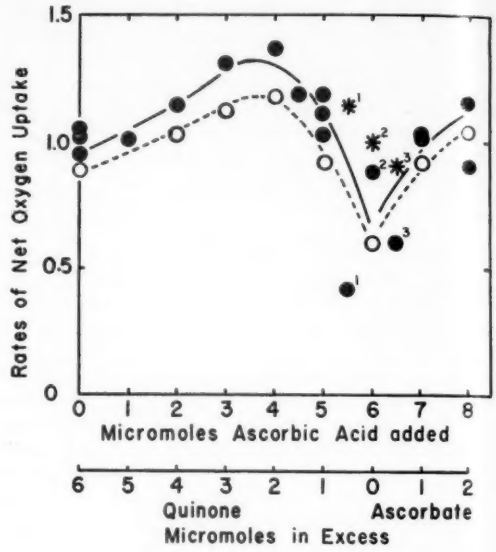
Results

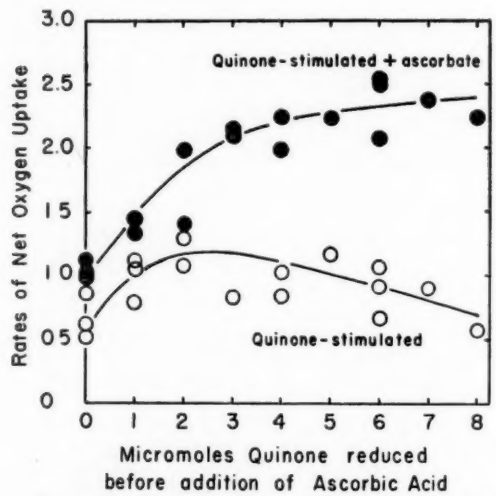
Photooxidation of Ascorbic Acid Coupled With the Mehler Reaction: I. *Effect of ascorbic acid concentration on rates of net oxygen uptake*: The addition of ascorbic acid to chloroplast reaction mixtures containing catalase and ethanol resulted in accelerated rates of net oxygen uptake in the light. When an excess of oxygen equivalent to the added ascorbic acid had been consumed, rates of oxygen uptake reverted to control values. The rates of oxygen uptake during photooxidation of ascorbic acid increased with the amount of ascorbic acid present at the beginning of illumination up to 4 μmoles per vessel (ca 10^{-3} M) (fig 1). With the chloroplast density used, further increases in ascorbic acid concentration resulted in no further increase in rates.

II. *Effects of quinone on rates of photooxidation of ascorbic acid by Mehler reaction mixtures*: Three variations of experimental procedure were used to test for effects of added ascorbic acid on rates of the Mehler reaction in the presence of quinone. In the first, quinone and ascorbic acid were mixed in the dark before adding chloroplasts, catalase, and ethanol. The amount of quinone added was held constant (6 μmoles) while the amount of ascorbic acid added to a series of vessels was varied (0–8 μmoles). The reaction between the ascorbic acid and quinone was completed before addition of the other components of the reaction mixture. It might be expected that only the stimulant present in excess would have an accelerating effect on the subsequent chloroplast reaction in the light. In each case it was possible to compare the rate of net oxygen uptake with rates observed in other experiments (in which aliquots of the same chloroplast preparation had been used) where only the equivalent amount of quinone *or* ascorbic acid was present in the reaction mixture. Both the observed rates for each mixture of quinone and ascorbic acid and the predicted rates are plotted in figure 2. In general, observed rates with the mixtures were slightly higher than those expected from experiments in which quinone or ascorbic acid alone was added to the reaction mixture. The greatest discrepancies were observed when equivalent or nearly equivalent amounts of quinone and ascorbic acid had been mixed. In these cases, two distinct rates were observed: the first, (a low rate) agreed with the rate expected from a reaction mixture containing neither quinone nor ascorbic acid; about 30 minutes after the beginning of illumination a second, higher rate attributable to ascorbate was observed (as much as twice the initial rate) which continued until oxygen uptake ceased at the end of the period of illumination. The discrepancy between observed and expected rates of net oxygen uptake may in part be explained by the action of hydroquinone. Mehler (19) reported a small stimulation of net oxygen uptake by this substance. Although a small stimulation of the Mehler reaction by hydroquinone was found in these experiments, its presence had no effect on the rates of oxygen uptake in reaction mixtures containing ascorbic acid (see below).

In a second variation of these experiments, ascorbic acid was added to the reaction mixture after the completion of quinone reduction. Quinone (in amounts varying from 0–8 μmoles) was equilibrated with chloroplasts, catalase, and ethanol in the dark. A fixed amount of ascorbic acid (6 μmoles) was tipped from the sidearm of each vessel after a period of illumination in which quinone reduction was completed and a period of quinone-stimulated Mehler reaction was followed. Rates of the quinone-stimulated Mehler reaction increased with increasing concentrations of quinone and were maximally stimulated in reaction mixtures in which 2 μmoles of quinone had been reduced in the light. These stimulated rates were approximately twice control rates. At higher quinone concentrations the rates of net oxygen uptake following quinone reduction showed marked inhibition







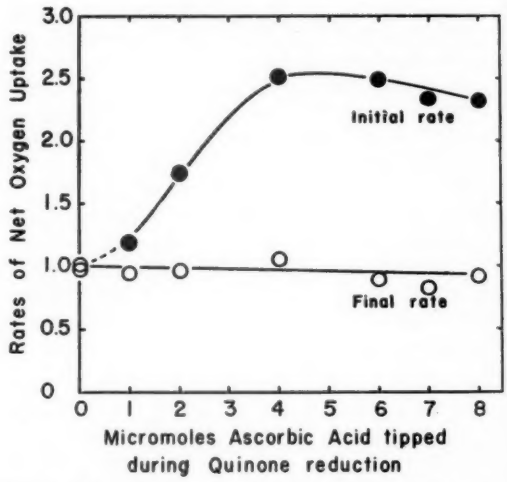


FIG. 1. (*upper left*). Relationship between amounts of ascorbic acid initially present in Mehler reaction mixtures and observed rates of net oxygen uptake (μl O_2/min/vessel). Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 0 to 8 μmoles ascorbic acid.

FIG. 2. (*upper right*). Rates of net oxygen uptake by Mehler reaction mixtures in which ascorbic acid and quinone were mixed before addition of chloroplasts. Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles quinone, 0 to 8 μmoles ascorbic acid. — ● — ● — Observed rates of net oxygen uptake in the light; — ○ — ○ — expected rates; ●¹, ●², ●³ initial rates; *¹, *², *³, final rates (μl/O_2/min/vessel). The initial upward swing represents a relief of the inhibition due to excess quinone; the first peak is due to the maximal quinone effect; and the second upward curve is due to the ascorbate effect. The dip in the middle represents the addition of dehydroascorbate plus hydroquinone which would not be expected to produce stimulation.

FIG. 3. (*lower left*). Rates of net oxygen uptake before and after addition of ascorbic acid to quinone-stimulated Mehler reaction mixtures (μl O_2/min/vessel). Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 0 to 8 μmoles quinone, 6 μmoles ascorbic acid (in sidearm).

FIG. 4 (*lower right*). Initial and final rates of net oxygen uptake in Mehler reaction mixtures following addition of ascorbic acid during quinone reduction (μl O_2/min/vessel). Vessel contents: 3 cc chloroplasts (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 8 μmoles quinone, 0 to 8 μmoles ascorbic acid.

and stimulated rates at the highest quinone concentrations were only slightly higher than the control rates. In all cases, the rates of net oxygen consumption increased after addition of ascorbic acid from the sidearms of the vessels and these rates increased with the amount of quinone which had previously been reduced by the chloroplasts in the light. Rates of net oxygen uptake after adding ascorbic acid were approximately equal to the rates of oxygen production during quinone reduction at concentrations of quinone above 3 μmoles per vessel. There were no further increases in rates of oxygen uptake in vessels which had initial concentrations of quinone higher than 3 μmoles per vessel (see fig 3). As a control experiment, from 0 to 6 μmoles of hydroquinone were substituted for quinone while the experimental procedure was otherwise the same as that described above. There was a slight stimulation of the Mehler reaction in the presence of hydroquinone, but pretreatment with hydroquinone did not enhance the rates of net oxygen uptake after addition of ascorbic acid to the reaction mixture. After the ascorbic acid tip, rates of net oxygen uptake were approximately twice unstimulated Mehler rates (as would be expected from ascorbic acid acting alone in the system).

In a third variation of the pattern of adding ascorbic acid and quinone, ascorbic acid was added during illumination, but before the completion of quinone reduction. Eight μmoles of quinone were equilibrated with chloroplasts, catalase, and ethanol in the dark. Oxygen production was measured for 25 minutes after the beginning of illumination and then from 0 to 8 μmoles of ascorbic acid in solution was tipped into each reaction mixture from the vessel sidearm. Production of oxygen stopped immediately and oxygen uptake began at rates which exceeded the preceding rates of oxygen production. The duration of these rapid rates of oxygen uptake was, of course, determined by the amount of ascorbic acid added and the amount of unreduced quinone remaining in the reaction mixture at the time of the tip. The final rate of oxygen uptake in each vessel was equal to that of the quinone stimulated Mehler reaction to which no ascorbic acid had been added (see fig 4).

PHOTOOXIDATION OF ASCORBIC ACID COUPLED WITH EXCHANGE REACTION: The Mehler reaction and the exchange reaction are basically the same: they differ only in the manner in which H_2O_2 formed by reduction of molecular oxygen is removed from the reaction mixture. In contrast to the Mehler reaction [in which H_2O_2 is removed through the coupled oxidation of ethanol to acetaldehyde (12)], in the exchange reaction H_2O_2 is dismuted to $H_2O + \frac{1}{2} O_2$ by the endogenous catalase in the chloroplasts (Brown & Good, 3). There is, therefore, no net oxygen uptake because of the exact balance between the oxygen consumed in reaction with the reduced product of photolysis and the production of one-half equivalent of oxygen from decomposition of this product by catalase plus one-half equivalent from the oxidized product of photolysis. Thus, it is not possible to measure the rate of an exchange reaction manometrically and this reaction can be followed only by the use of tracer oxygen. It seemed probable that ascorbic acid oxidation coupled to the exchange reaction would create an imbalance between consumption and production of oxygen which could be measured manometrically. This, in fact, was the case: addition of ascorbic acid to a chloroplast suspension with no other addenda resulted in a small, but measurable, uptake of oxygen in the light and (as in the Mehler reaction) this net uptake of oxygen was greatly enhanced when ascorbic acid was added to the reaction mixture following the reduction of quinone. It was possible, then, to vary the patterns of adding ascorbic acid and quinone and to measure the resulting net oxygen consumption manometrically.

TABLE I

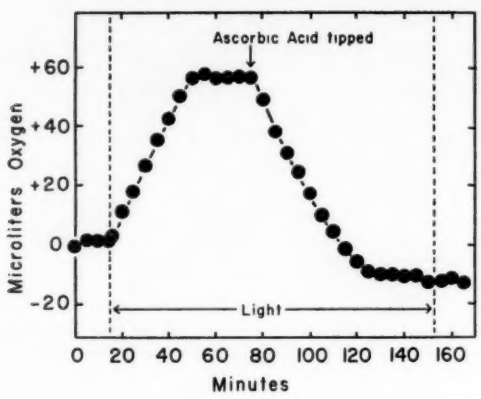
EFFECT ON EXCHANGE REACTION OF QUINONE & ASCORBIC ACID MIXED BEFORE ADDING CHLOROPLASTS

QUINONE/ VESSEL, μmoles	ASCORBIC ACID/VESSEL, μmoles	OXYGEN PRODUCED AFTER ASCORBIC ACID TIP, μl	
		OBSERVED	EXPECTED
6	0	64	67.2
6	2	36	44.8
6	4	25	22.4
6	6	0	0
6	8	0	0

Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 μmoles quinone, 0 to 8 μmoles ascorbic acid.

Table I summarizes the results of an experiment in which 6 μmoles of quinone and from 0 to 8 μmoles of ascorbic acid were mixed in the dark before adding the chloroplast suspension. The quantities of oxygen produced on illumination of the reaction mixture agreed closely with the amounts expected (based on the assumption of a quantitative reaction between ascorbic acid and quinone and the absence of any mechanism by which either of these substances could be regenerated). In the vessel containing equivalent amounts of quinone and ascorbic acid, no net uptake or production of oxygen was observed on illumination. In the presence of excess ascorbic acid, there was a slow uptake of oxygen in the light (−0.13 μl/min).

A rapid uptake of oxygen was observed when the addition of ascorbic acid followed the completion of a Hill reaction with quinone (see fig 5). Net oxygen consumption continued only as long as unoxidized ascorbic acid remained in the reaction mixture. The exchange reaction which continued after this point could not be measured manometrically. In a series of such experiments, from 0 to 10 μmoles of quinone were equilibrated with chloroplasts in the dark. After the completion of quinone reduction in the light, 6



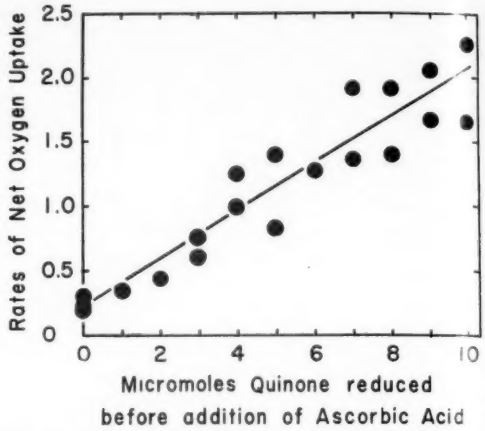


FIG. 5 (*left*). Representative time course of net oxyg n uptake: ascorbic acid added to quinone-stimulated exchange reaction. Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll) 6 μmoles quinone, 6 μmoles ascorbic acid (in sidearm).

FIG. 6 (*right*). Dependence of rate of net oxygen uptake during ascorbic acid oxidation on amount of quinone previously reduced by chloroplast preparations. Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 0 to 10 μmoles quinone, 6 μmoles ascorbic acid (in sidearms). Rates expressed as μl O_2/min/vessel.

μmoles of ascorbic acid were tipped from the sidearm of each vessel. As can be seen in figure 6, the rates of net oxygen uptake during photooxidation of ascorbic acid increased linearly over the range of amounts of quinone reduced prior to the addition of ascorbic acid to the system and showed no tendency to saturate.

In a second series of experiments, varied amounts of ascorbic acid (0–8 μmoles) were tipped from the sidearms of the reaction vessels during illumination, after oxygen production equivalent to approximately half reduction of 8 μmoles of quinone had been measured. The contents of the vessel sidearms were tipped without rinsing to add ascorbate as rapidly as possible and to keep protocol comparable to that of mass spectrometer experiments where rinsing was impossible. The expected production or consumption of oxygen after adding ascorbic acid was calculated assuming that 90 % of the contents of the sidearms had been transferred to the main compartment of each vessel. The results of these calculations and the measured volumes of net oxygen production or consumption are summarized in table II. Again there was close agreement between expected and observed values.

TABLE II

EFFECT ON EXCHANGE REACTION OF ASCORBIC ACID TIPPED DURING QUINONE REDUCTION

ASCORBIC ACID IN SIDEARM, μmoles	O_2 PRODUCED BEFORE ASCORBIC ACID TIP μl	TOTAL OXYGEN UPTAKE OR PRODUCTION AFTER ASCORBIC ACID TIP, μl	
		OBSERVED	EXPECTED
0	50	+40	+39.6
2	40	+21.5	+29.4
4	38	+ 8	+11.3
6	38	−20	− 9.1
8	38	−29	−29.0

Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 8 μmoles quinone, 0 to 8 μmoles ascorbic acid (in sidearms).

The rates of net oxygen uptake in the light due to unbalanced uptake and production in the exchange reaction were not dependent on the concentrations of ascorbic acid between 2 and 8 μmoles per vessel. In vessels containing ascorbic acid plus 3 cc chloroplast suspension, there were no detectable pressure changes in the dark, but on illumination there was measurable oxygen uptake: with 2 μmoles of ascorbic acid in the vessel the rate of oxygen uptake was 0.16 μl per minute, while with 8 μmoles of ascorbic acid in the vessel the rate of uptake was 0.13 μl per minute. There was no measurable pressure change in these vessels during a dark period following illumination. In vessels containing no ascorbic acid there were no net changes in pressure in the light or in the dark.

It had been assumed that the capacity for light-dependent oxidation of ascorbic acid is associated with the Hill activity of the chloroplast preparations which mediated these reactions. To test this assumption, the rates of these reactions with active chloroplasts were compared to rates with heat-inactivated preparations (heated for 2 hr at 75° C). Active chloroplasts were freshly thawed and diluted aliquots of the chloroplast preparations used for heat-inactivated controls. Even this relatively long heating did not completely destroy the capacity of these chloroplast preparations to carry out reactions of the Hill type, or to mediate the photooxidation of ascorbic acid (see table III). However, heat treatment did reduce all the activities

TABLE III

EFFECTS OF HEAT-INACTIVATION ON CHLOROPLAST ACTIVITY

CHLOROPLAST SYSTEM	QUINONE RATE, μl/min	% OF ACTIVE RATE	MEHLER OR EXCHANGE RATE, μl/min	% OF ACTIVE RATE	RATE AFTER ASCORBIC ACID TIP, μl/min	% OF ACTIVE RATE
			A. Quinone-Mehler + ascorbic acid			
Active	+1.48	100 %	−0.91	100 %	−3.32	100 %
	+1.46		−0.93		−3.23	
Heated	+0.12	0 %	...*	...*	−0.44	15 %
	−0.12				−0.56	
			B. Mehler + ascorbic acid			
Active	...	...	−1.53	100 %	−2.26	100 %
	...	...	−1.23		−1.94	
Heated	...	...	−0.09	0 %	−0.27	9 %
	...	...	0		−0.11	
			C. Quinone-stimulated exchange + ascorbic acid			
Active	+2.02	100 %	−0.41	100 %	−1.81	100 %
Heated	0.39	19 %	...*	...*	−0.20	11 %

* Unreduced quinone still in vessel.

Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 μmoles quinone (present in A & C), 6 μmoles ascorbic acid, 6 mg catalase (present in A & B), 0.1 cc 50 % ethanol (present in A & B).

measured to a small fraction of those of active chloroplasts, and these experiments support the hypothesis that the capacity of chloroplast preparations to mediate the photooxidation of ascorbic acid (whether or not quinone is added to the reaction mixture) is dependent on their Hill activity.

TRACER EXPERIMENTS TO DETERMINE NATURE OF ASCORBIC ACID EFFECTS: The manometric experiments described above demonstrated that light-dependent oxidation of ascorbic acid by chloroplast preparations resulted in accelerated oxygen uptake. It was necessary to utilize tracer techniques, however, to determine whether stimulated rates of net oxygen consumption after addition of ascorbic acid are independent of the products of photolysis, i.e. superimposed on the oxygen uptake and production associated with Mehler and exchange reactions; or whether ascorbic acid is oxidized by the oxidized product of photolysis (or another precursor of oxygen).

Aliquots of the same chloroplast preparation were used in all the experiments described below. Each reaction mixture contained 0.21 mg chlorophyll and experimental conditions such as concentration and volume of other components of the reaction mixtures, intensity of illumination, temperature, and oxygen tension were held as constant as possible. At least six replicate experiments were run of each of the following types: I. Addition of ascorbic acid to an unstimulated Mehler reaction; II. Addition of ascorbic acid to a quinone-stimulated Mehler reaction, and III. Addition of ascorbic acid to a quinone-stimulated exchange reaction. In all cases, ascorbic acid was added during the period of illumination and after stable rates of light-dependent oxygen consumption and production had been measured for at least 20 minutes.

I. ADDITION OF ASCORBIC ACID DURING UNSTIMULATED MEHLER REACTION: The time course of one representative experiment of this type [showing partial pressure changes of ordinary oxygen (mass 32) and tracer oxygen (mass 34)] is illustrated by figure 7. The average rates calculated from six such experiments are summarized in table IV. These ex-

TABLE IV

EFFECTS OF ADDING ASCORBIC ACID TO UNSTIMULATED MEHLER SYSTEM

	UPTAKE, μl/min	PRODUCTION, μl/min	NET, μl/min
Unstimulated Mehler rates	−1.33 ± 0.33*	+0.64 ± 0.14	−0.75 ± 0.24
Rates after adding ascorbic acid	−1.96 ± 0.18	+0.72 ± 0.14	−1.25 ± 0.18
% of rate before adding ascorbic acid	148 %	113 %	167 %

* Mean of six determinations ± standard deviation of mean.

Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles ascorbic acid (in sidearms).

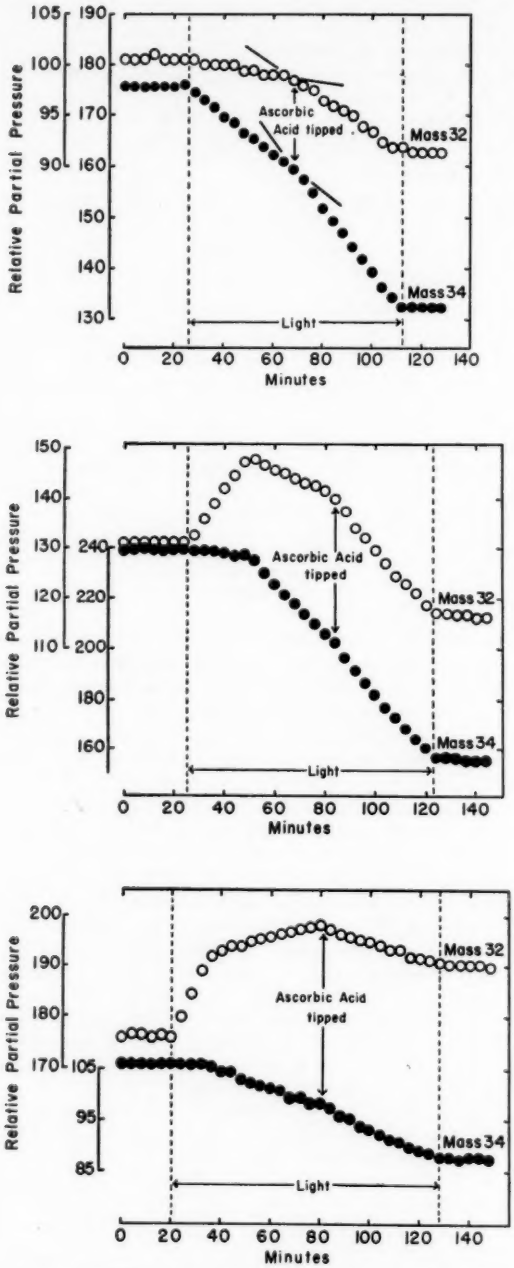


FIG. 7. Effect of addition of ascorbic acid on time courses of normal and tracer oxygen in the Mehler reaction. Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles ascorbic acid (in sidearm). To convert partial pressure units of the ordinate to microliters oxygen multiply by 3.02 for mass 32, by 1.00 for mass 34.

FIG. 8. Time courses of normal and tracer oxygen during quinone reduction, quinone-stimulated Mehler reaction and photooxidation of ascorbic acid. Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles quinone, 6 μmoles ascorbic acid (in sidearm). To convert partial pressure units of the ordinate to microliters oxygen multiply by 3.27.

periments showed that increases in rates of net uptake of oxygen after adding ascorbic acid to Mehler reaction mixtures resulted from two changes in the pattern of oxygen metabolism: rates of both uptake and production of oxygen were accelerated. After adding ascorbic acid to the reaction mixture, rates of oxygen production increased by 13 % while rates of oxygen consumption increased by 48 %. Because rates of both uptake and production of oxygen were changed (rather than only the rate of oxygen consumption) it is more probable that ascorbic acid is oxidized via the intermediary reactions of the products of photolysis, rather than via chloroplast-mediated oxidation which is independent of the Hill activity of the chloroplasts.

II. ADDITION OF ASCORBIC ACID DURING QUINONE-STIMULATED MEHLER REACTION: In this group of experiments quinone was equilibrated in the dark with the components of the Mehler reaction mixture to insure maximum quinone stimulation (8). The time course of a representative experiment of this group is shown in figure 8. At the beginning of illumination, there was an increase in the relative partial pressure of mass 32, while there was no change in partial pressure of mass 34. This indicated that only quinone was being reduced by the reduced product of photolysis. As soon as the reduction of quinone was complete, net uptake of both mass 32 and mass 34 began. Adding ascorbic acid to the reaction mixture resulted in a marked increase in the rate of uptake of mass 32. By comparison, the change in slope of the mass 34 curve was very small. After addition of ascorbic acid, rates of net oxygen uptake increased an average of 91 %. This change in net rate of oxygen uptake was the consequence of a 32 % increase in oxygen uptake and a 46 % decrease in the rate of oxygen production (see table V). Once again, the more plausible mechanism of ascorbic acid oxidation was its reaction with the oxidized product of photolysis or some intermediate in the pathway to oxygen production.

III. ADDITION OF ASCORBIC ACID DURING QUINONE-STIMULATED EXCHANGE REACTION: As in the previous set of experiments, quinone was equilibrated with chloroplasts in the dark. At the beginning of

FIG. 9. Time courses of normal and tracer oxygen during quinone reduction, quinone-stimulated exchange, and photooxidation of ascorbic acid. Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 μmoles quinone, 6 μmoles ascorbic acid (in sidearm). To convert partial pressure units of the ordinate to microliters oxygen multiply by 2.86.

TABLE V

EFFECTS OF ADDING ASCORBIC ACID TO QUINONE-STIMULATED MEHLER SYSTEM

	UPTAKE, μl/min	PRODUCTION, μl/min	NET, μl/min
Quinone-stimulated Mehler rates	−3.19 ± 0.19*	+1.42 ± 0.21	−1.79 ± 0.08
Rates after adding ascorbic acid	−4.20 ± 0.28	+0.77 ± 0.15	−3.42 ± 0.21
% of rate before adding ascorbic acid	132 %	54 %	191 %

* Mean of six determinations ± standard deviation of mean.
Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles quinone (equilibrated in dark with reaction mixture), 6 μmoles ascorbic acid (in sidearms).

illumination, partial pressure changes characteristic of quinone reduction were observed (for time course of a typical experiment see fig 9). On completion of quinone reduction, the balanced oxygen uptake and production of the exchange reaction began. In this reaction the rate of oxygen uptake (due to reduction of oxygen by the reduced product of photolysis) is just balanced by the production of one-half equivalent of oxygen by the action of endogenous catalase on the peroxide thus formed and a second one-half equivalent from the oxidized product of photolysis. Both mass 32 and mass 34 are consumed in the initial reaction with the reduced product of photolysis. Oxygen produced by the action of endogenous catalase is composed of mass 32 and mass 34 in the proportions of their occurrence in the oxygen consumed; the oxygen produced from the oxidized product of the splitting of water is almost entirely mass 32 because of the overwhelming preponderance of H_2O^{16} over H_2O^{18} in the milieu. Hence, during the exchange reaction (in which there is no net change in the partial pressure of oxygen) there is an increase in the relative partial pressure of mass 32 and a decrease in the relative partial pressure of mass 34. On addition of ascorbic acid there was a change from net production to net uptake of mass 32 accompanied by a slight increase in rate of net uptake of mass 34. The average rates for six replicate experiments are summarized in table VI. These data provide the most convincing proof of the hypothesis that ascorbic acid is oxidized via the oxidized product of the splitting of water: the rate of oxygen production decreased by 47 % on addition of ascorbic acid to the reaction mixture. This decrease in oxygen production from the oxidized product of the splitting of water is apparent from the marked change in slope of mass 32 (from production to consumption). Uptake of mass 34 was only slightly accelerated following the ascorbic acid tip.

DISCUSSION

Viewed as a whole, these experiments show that accelerated net oxygen uptake by illuminated chloroplasts on addition of ascorbic acid is the result of two simultaneous changes, i.e. the change in net rate is the result of changes in both uptake and production of oxygen. The unequal effects of ascorbic acid on rates of uptake and production can be explained by a single reaction mechanism: the oxidation of ascorbic acid by an intermediate on the oxidized side of photolysis. This diversion of a portion of one of the products of the splitting of water does not completely block the production of oxygen, and thus there is evidence of a competition between ascorbic acid and the normal enzymatic pathway to oxygen. The proposed mechanism explains both the increased rates of oxygen uptake observed in all cases and the accelerated rates of oxygen production on addition of ascorbic acid to an unstimulated Mehler reaction. If the products of photolysis are produced more rapidly than they can be transported along their normal enzymatic pathways, then either of the following conditions would result: A. A back reaction (or recombination of the oxidized and reduced products of the splitting of water) could remove an appreciable fraction of these products from the system; or B. If only one part of the enzyme system were limited, one of the products of photolysis or a subsequent intermediate might accumulate. Oxidation of ascorbic acid by the oxidized product of the splitting of water could affect either of the above conditions: more efficient removal of this product would decrease the

TABLE VI

EFFECT OF ADDED ASCORBIC ACID ON QUINONE-STIMULATED EXCHANGE

	UPTAKE, μl/min	PRODUCTION, μl/min	NET RATE, μl/min
Quinone reduction	−0.12 ± 0.18*	+2.97 ± 0.27	+2.86 ± 0.33
Quinone-stimulated exchange	−2.69 ± 0.39	+2.48 ± 0.26	−0.11 ± 0.08
After adding ascorbic acid	−3.62 ± 0.39	+1.32 ± 0.16	−1.15 ± 0.16
% of rate before adding ascorbic acid	135 %	53 %	...

* Mean of six determinations ± standard deviation of mean.
Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 μmoles quinone (equilibrated in dark with reaction mixtures), 6 μmoles ascorbic acid (in sidearms).

probability of back reaction by removing from the reaction system any accumulated intermediates in the pathway to oxygen. Acceleration of the oxygen-consuming reaction in all three of the systems studied (unstimulated Mehler reaction, quinone-stimulated Mehler reaction, & quinone-stimulated exchange reaction) indicates that the extent of back reaction of the products of photolysis is reduced. The rate of oxygen production may be calculated from an observed rate of oxygen consumption according to the usual stoichiometry (20). In all three experimental conditions the rate of oxygen production relative to the observed O_2 uptake is decreased by ascorbate. Even in the unstimulated Mehler reaction (where rates of oxygen production actually increased on addition of ascorbic acid), the stoichiometry of the reaction was changed, indicating that some oxygen precursor was being diverted during the oxidation of ascorbic acid.

It has not been established if the light-dependent oxidation of ascorbic acid by chloroplasts is an enzymatic reaction. The absence of a normal type of ascorbic acid oxidase is certainly apparent. Both manometric and tracer oxygen measurements indicated that oxidation of ascorbic acid was in all cases a light-dependent reaction: it occurred only when chloroplast preparations were illuminated and stopped immediately when lights were turned off. It is possible that chloroplasts contain an ascorbic acid photooxidase but it is not necessary to postulate its existence in order to explain the experimental results.

The stimulatory effects of quinone were discussed in a previous paper (8). In the light of recent isolation of naturally-occurring quinones from chloroplasts by Bishop (2) and Crane (6) and the demonstration that such *p*-quinone derivatives are necessary for Hill activity, it is not surprising that quinone should enhance the overall activity of chloroplast reaction systems. Tracer experiments have demonstrated that quinone stimulation does not change the stoichiometry of the Mehler or exchange reactions (8): in both reactions the ratios of uptake to production of oxygen remained the same as those observed in unstimulated reactions. It is possible that the naturally-occurring quinones (or benzoquinone added to the reaction mixture) are directly responsible for ascorbic acid oxidation. Light and Hill activity of the chloroplasts would be required by this reaction mechanism for oxidation of ascorbic acid via a cyclic reoxidation of reduced quinone by the oxidized product of photolysis.

Summary

The light-dependent oxidation of ascorbic acid by chloroplast preparations was investigated using manometric and tracer techniques. Such photooxidations of ascorbic acid by Mehler or exchange reaction mixtures result in increased rates of net oxygen uptake. Both the Mehler and exchange reactions are accelerated by previous reduction of quinone, and their activity in the mediation of ascorbic acid oxidation is similarly enhanced following quinone reduction. Experiments with tracer oxygen indicate that increased net oxygen consumption after adding ascorbic acid to chloroplast reaction systems is the consequence of simultaneous increase in oxygen consumption and decrease in oxygen production. According to the postulated reaction mechanism, oxidation of ascorbic acid results from a reaction with the oxidized product of photolysis via a cyclic oxidation and reduction of either endogenous quinones or benzoquinone added to the reaction mixture.

Acknowledgments

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VARIATIONS IN STARCH & TOTAL POLYSACCHARIDE CONTENT OF PINUS PONDEROSA NEEDLES WITH FLUORIDE FUMIGATION[1]

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INTRODUCTION

Stoklasa (10) reported that leaves of plants showing symptoms of chronic sulfur dioxide injury have a lower starch content than normal leaves. Haselhoff et al (4) suggested that lowered starch content was associated only with chronic sulfur dioxide injury, since, in the instance of acute injury, the starch level is fixed at the level which existed at the time of the cell destruction. This hypothesis was not experimentally substantiated. Swain (11) stated that repeated mild, daily, non-marking sulfur dioxide fumigations resulted in no reduction in carbohydrate content of the leaves. Katz and Pasternack (6) studied the effect of concentrations of sulfur dioxide insufficient to produce leaf markings and found an increase in sulfur content, but no significant change in starch concentration.

No similar studies have been found in the literature relating (a) starch and polysaccharide levels in plant leaves with (b) fluoride fumigation. This work was undertaken to provide information on the relation between (a) starch and polysaccharide content of Pinus Ponderosa needles and (b) fluoride exposure level and sequence of fumigation.

EXPERIMENTAL

FLUORIDE FUMIGATION PROCEDURES: Five groups of ponderosa pine (*Pinus ponderosa* Laws) were

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used, each consisting of six to ten separate trees ranging from four to eight years of age. The trees were grown in 5 gallon cans and fumigated in plastic greenhouses under conditions which have been reported (1).

Group I was the control set and was divided into three sub-groups. Subgroup A was placed in the control greenhouse 8 hours per day, 5 days per week; Subgroup B was placed in the control house 8 hours per day, 2 days per week; and Subgroup C was placed in the control house 4 hours per day, 2 days per week. Groups II and III were fumigated 8 hours per day, 5 times weekly, at 0.5 and 1.5 μg F^-/cu m, respectively. Group IV was fumigated 8 hours per day, twice weekly, at 5 μg F^-/cu m. Group V was fumigated 4 hours per day, twice weekly, at 10 μg F^-/cu m. Trees were maintained out-of-doors when not in one of the greenhouses.

Reagents: Perchloric Acid: 72%, reagent grade. Iodine—potassium iodine: Grind 7.5 g iodine and 7.5 g potassium iodide with 150 ml of water, dilute to 250 ml, and filter through no. 3 Whatman paper with suction. Potassium iodide: 0.4 N. Dissolve 66.408 g of potassium iodide in distilled water and dilute to 1,000 ml. Potassium iodate: 0.005 N. Dissolve 1.07 g of potassium iodate in distilled water and dilute to 1,000 ml. Alcoholic sodium hydroxide: Dilute 350 ml ethanol, 100 ml water, and 25 ml 5 N sodium hydroxide to 500 ml of water and filter. Anthrone solution: Dissolve 1 g of anthrone in 500 ml of ethyl acetate (7). Sulfuric acid: 80%. Dilute 400 ml sulfuric acid 98% to 500 ml with water.

Analytical Procedures: *I. Sample Preparation*: Composite samples of approximately 30 grams each of the current year's needles were obtained weekly from each group of fumigated and control ponderosa pine trees. All samples were immediately dried for 24 hours at 65° C in a forced air oven. The dry needles were ground in a Waring blendor and stored for analysis.

II. Extraction of sugars: A 2 g sample of the finely ground, air dried pine needles was placed in a Soxhlet extractor and extracted for 48 hours with 80% ethyl alcohol to remove the simple sugars (8). The alcoholic solution was decanted and discarded. The sample was then extracted for 3 hours with ether to complete the removal of alcohol. The sample was air dried and the per cent extractable material present in the original sample was determined by weight difference.

III. Extraction of Acid—hydrolyzable Polysaccharides (starch): One-half gram of dried, alcohol-extracted solids was weighed into a 150 ml beaker and 8 ml of distilled water added. The mixture was heated to boiling and then cooled. The beaker was placed in an ice-water bath and 6 ml of 72% perchloric acid was added, stirring constantly. The extraction was continued for 1 hour with occasional stirring. The extract was vacuum-filtered through a sintered glass funnel, collecting the filtrate. The residue was washed several times with water, and the filtrate and washings were combined. The extraction was repeated, using 6 ml of water and 4 ml of perchloric water. All filtrates and washing were then combined (8).

IV. Determination of Starch: Forty-three ml of the perchloric acid extract were transferred to a 50 ml volumetric flask. Two ml of 0.4 N KI and 5 ml of 0.0005 N KIO_3 were added and well shaken. The absorbance of the blue color was determined with a spectrophotometer at 660 mμ after 15 minutes (8). The absorptivity was converted to mg of starch by reference to the standard curve.

V. Determination of total polysaccharide: The original perchloric acid extract was diluted 1:10 with distilled water. Two ml of the diluted extract were placed in a 10 ml volumetric flask and 0.05 ml of 2% anthrone in ethyl acetate was added. Then 5 ml of 98% sulfuric acid were added and well shaken. The flask was allowed to stand for 10 minutes. The reaction mixture was cooled to room temperature and diluted to 10 ml with 80% sulfuric acid. The light absorbance was spectrophotometrically determined at 630 mμ (7, 8). The absorptivity was converted to weight of polysaccharide by reference to the calibration curve.

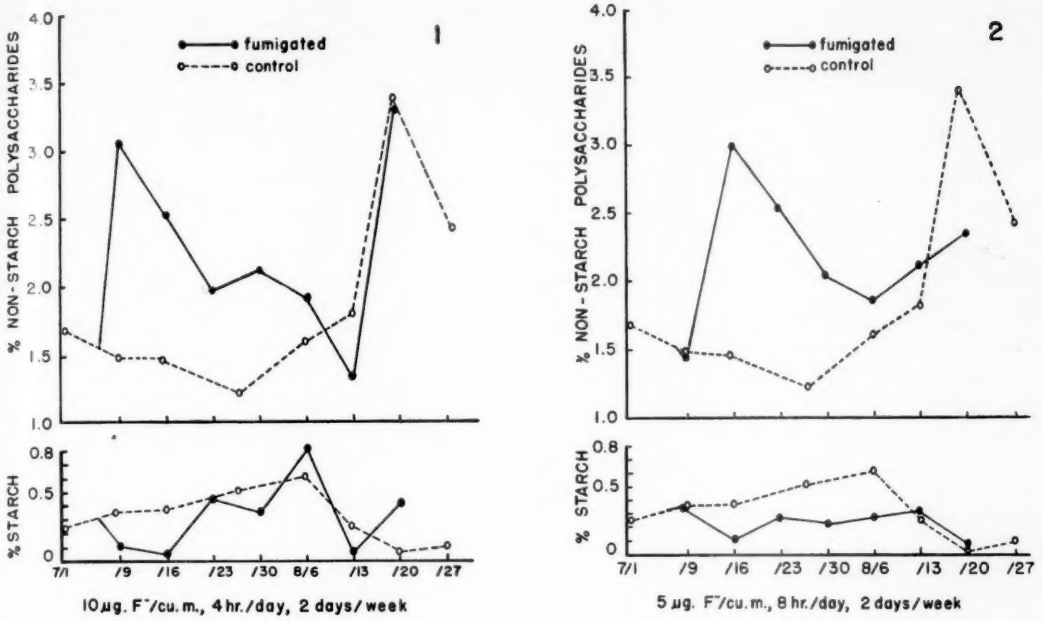
VI. Preparation of Standard Curves: An aliquot of a perchloric acid extract of a known weight of soluble starch was transferred to a centrifuge tube and 5 ml of 20% NaCl and 2 ml of the iodine–potassium iodide reagent were added. The solution was well mixed and allowed to stand for at least 20 minutes. The tube was centrifuged and the supernatant decanted with extreme care to avoid loss of precipitate. The precipitate was washed with 5 ml of alcoholic sodium chloride by gently shaking the tube, centrifuging, and decanting the liquid.

The precipitated starch was then converted to starch by adding alcoholic sodium hydroxide to the tube. The tube was gently shaken and tapped until all of the blue color was discharged. A stirring rod must not be used. Aliquots of the resultant solution were used to establish the standard polysaccharide curve with the anthrone reagent and the starch curve by means of the iodine blue color.

VII. Fluoride Analysis: One gram of air-dried and ground pine needles was slurried with CaO and distilled water for fluoride analysis. The slurry was first dried and partially ashed under infra-red heat lamps and finally ashed in a furnace at 600° C for 30 minutes. The ash was then fused with NaOH (9) and distilled from perchloric acid (12). The distillate was titrated by a modified high-salt-thorium nitrate method (2, 13).

Results

Pine needles were sampled and analyzed each week for starch, total polysaccharide, ethanol extractable material, and fluoride content. Unfortunately, it be-



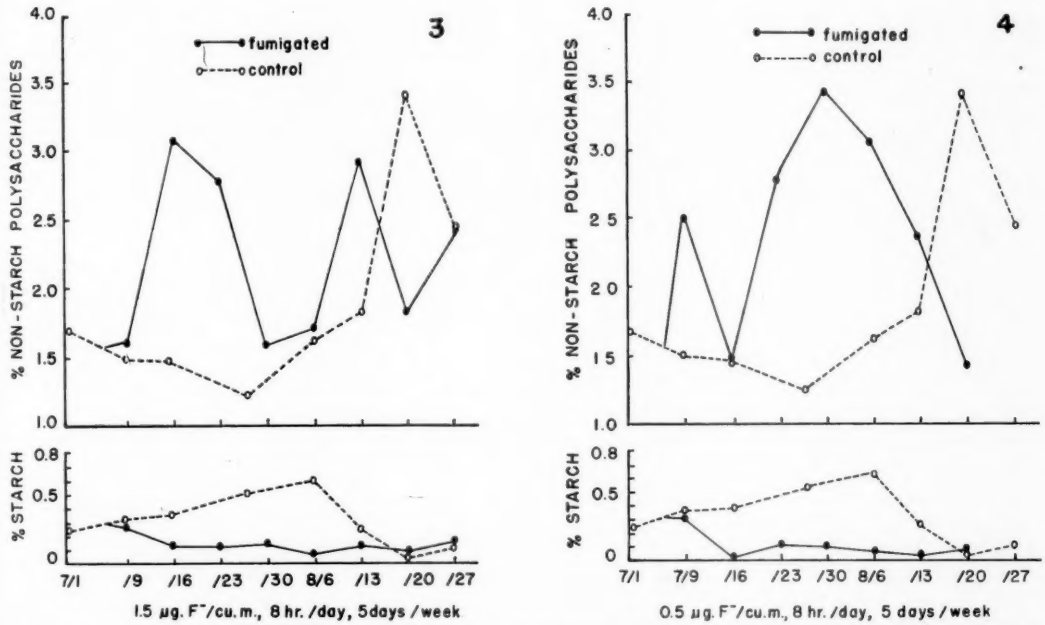


FIG. 1. Starch and non-starch polysaccharide content of fumigated and control pine needles—10 µg F^-/cu m, 4 hr/day, 2 days/week.

FIG. 2. Starch and non-starch polysaccharide content of fumigated and control pine needles—5 µg F^-/cu m, 8 hr/day, 2 days/week.

FIG. 3. Starch and non-starch polysaccharide content of fumigated and control pine needles—1.5 µg F^-/cu m, 8 hr/day, 5 days/week.

FIG. 4. Starch and non-starch polysaccharide content of fumigated and control pine needles—0.5 µg F^-/cu m, 8 hr/day, 5 days/week.

came necessary to discontinue sampling for fluoride analysis following the 3rd week of fumigation so as to reduce the rate of denuding of the test trees. Figures 1 to 4 show the time course of starch and of non-starch polysaccharide content of the ponderosa pine needles fumigated at the four levels of hydrogen fluoride compared with control needles.

These data indicate that A, the concentrations of non-starch polysaccharides were generally inversely proportional to the starch levels in all groups of fumigated trees: B, the starch levels in all fumigated groups declined whereas the non-starch polysaccharides increased rapidly following the initial week's fumigation; C, starch levels in the trees fumigated at the two highest fluoride levels twice weekly equaled or surpassed the starch levels found in the control trees toward the end of the fumigation sequence, whereas the starch levels found in the trees fumigated at the two lowest levels 5 days per week tended to remain at a uniform reduced level throughout the entire period.

Discussion

Comparison of the total fluoride to which each of the four groups of trees was exposed each week may be made using the concept of exposure factor (1). The exposure factor is defined as the arithmetic product of A, the atmospheric concentration of HF, B, the hours per day of exposure and C, the number of days per week of exposure. Thus Group II (0.5 μg F^-/cu m, 8 hours a day, 5 days/week) received an exposure of 20, Group III (1.5 μg F^-/cu m, 8 hours a day, 5 days/week) received an exposure of 60, Group IV (5 μg F^-/cu m, 8 hours a day, 2 days/week) received an exposure of 80, and Group V (10 μg F^-/cu m, 4 hours a day, 2 days/week) received an exposure of 80.

On the basis of the data presented in figures 1 to 4 it appears that one possible action of fluoride upon the polysaccharide system of the pine needles may be to alter the relationship between the non-polysacharides and starch. The data further indicate the possibility that plants may adapt themselves to concentrations of atmospheric fluorides in the range of 5 to 10 μg F^-/cu m if provided a recovery period between each exposure. Conversely plants may not so readily adapt to lower total, more nearly continuous fluoride fumigations. Similar observations of the relative effects of daily fumigations at the lowest fluoride concentration (1.5 μg F^-/cu m) as compared with the highest concentrations (5 & 10 μg F^-/cu m) on a twice weekly fumigation basis were previously observed when comparing the onset of initial leaf changes at three levels of fluoride concentration (1).

This apparent ability of plants to adapt to or show somewhat greater resistance to intermittent higher fluoride fumigation levels becomes significant in view of recent evidence that such intermittent fluoride fumigations are the type which do in fact exist in the field (3 & Adams & Koppe, 1960, unpubl. information). This is in contrast to the long time, low concentration average atmospheric concentrations (less than 1 μg F^-/cu m) which have previously been assumed to exist by many workers in this field (5).

These data re-emphasize the concept that the sequence of fluoride exposure may outweigh the influence of the actual fluoride level within a fumigation concentration range of 0.5 to 10 μg F^-/cu m.

Summary

Fluoride fumigation of *Pinus ponderosa* Laws resulted in an initial departure of the concentrations of starch and non-starch polysaccharides from similar levels in control tissue. It appeared that one possible mode of fluoride action was to alter the relationship between the non-starch polysaccharides, and starch. The data also indicate that ponderosa pine tend to adapt to fluoride fumigations at higher levels, 5 to 10 μg F^-/cu m, when the exposure is intermittent. This is in contrast to the reaction of the plant to lower fumigation concentrations, 0.5 and 1.5 μg F^-/cu m, of longer and more frequent duration and lower exposure factor. This apparent ability of pine to adapt to somewhat higher but more intermittent fumigation exposures becomes significant in relation to recently obtained information showing that intermittent fumigations do exist in the field (3). It had previously been assumed that field fumigations were of the long-time, low average concentration type (5).

The variation-range for the concentration of starch and for that of non-starch polysaccharide was not statistically different for the fluoride-fumigated plants and in the controls. Thus foliage analysis for starch and total polysaccharide levels could not be used to determine whether any particular field-grown plant had been exposed to a fluoride fumigation.

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INFLUENCE OF REPRODUCTIVE ORGANS ON SECRETION OF SUGARS IN FLOWERS OF STREPTOSOLEN JAMESONII, MIERS[1,2]

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Nectar secretion in nectaries of flowers pollinated by insects or birds generally coincides with pollen maturation, and as a result the nectar forager picks up mature pollen on its body. Foraging insects which have been studied exhibit a considerable degree of species constancy (16), and so the viable pollen of one flower is deposited on stigmas of other flowers of the same species. As the stigma is receptive to pollen at this time, the opportunity for fertilization is provided. Secretion by floral nectaries ceases with fertilization and senescence of the corolla (2). In unpollinated flowers secretion persists for a longer time.

In view of these relationships one might suspect the existence of a coordinating mechanism, possibly hormonal in nature, between the events culminating in pollen maturation and those leading to nectar secretion. An interesting example of an apparent correlation may be observed in nasturtium, (Tropaeolum sp.), in which part of the corolla forms a spur containing the nectary. Prior to pollen maturation, the filaments are curved away from the side of the flower which carries the spur. As the anthers dehisce, the filaments straighten so that their anthers are directly above the mouth of the spur. Nectar secretion begins at this time. Foragers seeking nectar in the spur, of necessity come into contact with the anthers and pick up mature pollen.

In a study of nectar secretion in plants with unisexual flowers, Fahn (4) found considerable differences between the nectar yields of staminate and pistillate flowers. The sex of the higher yielding flowers varied with the species. These observations suggested a relationship between the stamens and pistils and nectar secretion. The present work relates to the influence of the anthers and stigma on secretion in floral nectaries of *Streptosolen jamesonii*, Miers. In this species the flowers are perfect and the nectary forms part of the ovary. As nectaries and stigmas of some species have structural and functional features in common (3), the nectary and stigma of Streptosolen were compared with respect to their anatomy and secretory activity.

In addition to the outward movement of sugar in nectar secretion, the reverse movement was examined. Since Bonnier's suggestion (2) that nectar is reabsorbed by the flower after fertilization, indirect evidence of reabsorption, based on slight increases in total nectar yield with periodic removal of nectar (15) has been obtained. Recently Pedersen et al (14) have provided direct evidence of reabsorption by supplying C^{14}-labelled sucrose to pollinated alfalfa florets. Radioactivity was found throughout the plant. Absorption was not tested in unpollinated flowers. Ziegler and Lüttge (24) have demonstrated absorption of C^{14}-labelled glutamic acid by secreting nectaries of *Daucus carota*, *Abutilon striatum*, and *Viburnum opulus*.

Materials & Methods

Plant Material. *Streptosolen jamesonii* Miers is a shrub of the Solanaceae family which flowers profusely over several weeks and produces nectar in abundance. Nectar secretion commences around the time the flower opens and continues for about three

[1] Received revised manuscript January 3, 1961.
[2] Contribution of the Department of Apiculture. This work has represented a part of the programs of the Ontario Legume & Tree Fruit Research Committees.

days. Anther dehiscence coincides with the beginning of secretion. As the flower matures its color changes from yellow through orange to scarlet. The age of the flower can be estimated quite accurately from the color and diameter of the corolla.

The nectary is in the form of a ring around the base of the ovary. Nectar accumulates in the corolla tube. Much of the stigmatic surface consists of secretory tissue which produces a considerable volume of exudate. There are two pairs of stamens; the anthers of the upper pair are situated above the stigma and those of the lower pair below the stigma. In the greenhouse, pollen does not germinate on the stigma surface if the flower is not disturbed. The upper surface of the stigma, which is in a position to receive pollen from the upper anthers via gravity, remains dry; the lower surface, from which the exudate is secreted, cannot receive pollen through gravitational influence alone. The possibility of an effect on secretion of hormones produced by germinating pollen (5, 9, 10, 13) was thus ruled out.

All experimental plants were propagated clonally from a single parent plant.

Measurement & Analysis of Nectar & Stigma Exudate. Nectar was extracted and measured by the centrifuge method (22) or, when several collections were made from individual flowers, with glass capillaries of known volume. Concentration of total solids in the nectar (essentially sugars) was measured on the sugar scale of an Abbe refractometer. Nectar yields were recorded as weights of nectar sugar, calculated from volume and concentration data. Sugar weights, unlike volume and concentration values, are independent of post-secretion changes caused by evaporation of water from nectar in an atmosphere of low humidity (17). Yields of stigma exudate were estimated by weighing stigmas before and after washing with 70 % ethanol.

Ascending paper chromatography was used to identify the constituent sugars of nectar and stigma exudate. Equal weights of the dissolved solid materials in nectar and exudate were spotted on Whatman no. 1 paper together with sucrose, glucose, and fructose standards. Chromatograms were developed in *n*-butanol, ethanol, and water (4 : 2 : 1, v/v/v) and sprayed with *p*-anisidine-HCl (18). The ratios of reducing sugars to sucrose in nectar and exudate were estimated by the Somogyi method (21) before and after hydrolysis with 5 % HCl (7).

Extraction & Chromatography of Growth Substances From Stigmas. Stigmas were collected from flowers which had been open for approximately 24 hours, stored at −15° C until a sufficient number had been obtained, and extracted with peroxide-free ether. The extract was partitioned with a 4 % bicarbonate solution and the latter was acidified to pH 3 and extracted with ether. The final ether extract, containing the acidic ether-soluble substances, was evaporated to dryness. A portion of the residue was chromatographed on Whatman no. 4 paper for 12 hours at 20° C in various solvent mixtures for partial characterization. As 3-indoleacetic acid had been found in the styles of *Nicotiana tabacum*, a related species (9, 10), synthetic IAA was included in the chromatograms for reference purposes. Chromatograms were examined in ultraviolet light and sprayed with Salkowski reagent. Larger quantities of the residue were chromatographed on Whatman 3MM paper. The separated components were eluted with 80 % ethanol, the eluates were evaporated to dryness, and the residues were incorporated in lanolin for assaying of activity. Assays were also made with lanolin pastes of the crude acidic ether-soluble and neutral ether-soluble fractions.

Experimental Treatment of Flowers for Nectar Measurement. The course and duration of the secretion of nectar and stigmatic exudate were recorded in untreated flowers. Nectar yields were also measured in flowers receiving the following treatments in situ: A. removal twice daily of nectar from the corolla tube: B. excision of immature anthers 6 to 12 hours before the flowers opened: C. excision of the stigmas at the time the flowers opened, and D. application of a lanolin paste of synthetic growth regulators or ether-soluble materials extracted from the stigmas to the decapitated styles. Within each replicate flowers were carefully matched for age, size, and position on the plant. The nectar was collected when the flowers had reached maturity. Flowers showing any evidence of injury from handling were discarded.

Studies of Absorption & Movement of C^{14}-labelled Sucrose in Pistil. These experiments were designed to test reabsorption of nectar sugar at various stages of flower maturation, the influence of the anthers and stigma on reabsorption, translocation of reabsorbed sugar to the stigma and its secretion in the stigma exudate, and reabsorption of stigma exudate and secretion of its component sugars in nectar. As sucrose was found to be the major sugar in Streptosolen nectar (see below), C^{14}-sucrose (uniformly labelled) was used as a model. The nectar was removed from the corolla tubes with fine capillaries and replaced with 3 μl of a dilute solution of C^{14}-sucrose of approximately one μc activity in the following groups of flowers: A. young flowers which had just opened; B. mature flowers, which had been secreting for about 48 hours; C. senescent flowers, which had been open for about 96 hours and in which secretion had ceased; D. mature flowers from which the anthers had been removed 6 to 12 hours before opening, and E. mature flowers from which the stigmas had been removed immediately after opening. After 24 hours exposure to the radioactive sucrose the pistils were removed and washed thoroughly with 70 % ethanol. The stigma exudate was collected from group B. Some pistils from each group were radioautographed on Kodak dental X-ray film for 2 to 12 days. For quantitative measurements of radioactivity individual ovaries, styles, and stig-

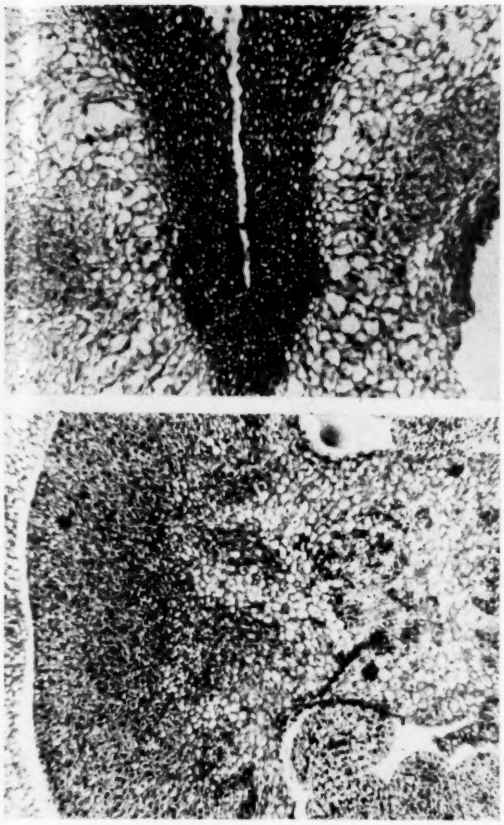

FIG. 1. Cross-sections through the secretory tissues of the stigma and nectary of the Streptosolen flower.

Stigma (*above*): the secretory tissue is the area of small closely packed, heavily stained, cells in the middle of the section.

Nectary (*below*): the heavily stained cells on the left are a sector of the nectary.

mas of five flowers of each group (with the exception of the group without stigmas) were extracted for several hours with hot 80 % ethanol, the extracts were plated on planchets, and counts were made with a GM flow counter (1).

The stigmatic exudate was chromatographed, and the sections of the paper containing the component sugars were radioautographed.

Absorption of sugar by the stigma was tested by placing microliter quantities of C^{14}-sucrose of 1 μc activity on the secretory surface of stigmas of newly opened flowers. As glucose and fructose occur in greater quantity in the exudate one of these would have been preferable to sucrose as a model, but it was not immediately available. After 24 hours nectar was collected and tested for radioactivity with a flow counter. Radioautographs were made of filter paper sections of chromatographically separated component sugars.

RESULTS

Cross-sections of secretory tissue from stigma and nectary in figure 1 show closely-packed, protoplasm-rich cells with large nuclei in both tissues, and figure 2 shows concurrent periods of secretory activity. The compositions of the respective secretions differ, however. Chiefly sucrose, with appreciable quantities of glucose and fructose, appeared in nectar chromatograms. Sucrose acounted for 64.3 ± 2.3 % and reducing sugars for 32.2 ± 0.2 % of the total solids in nectar (mean of 4 Somogyi determinations). Chromatograms of stigma exudate showed chiefly glucose and fructose, with traces of sucrose. Reducing sugars constituted roughly 60 % and sucrose 10 % of the dissolved solids in the exudate. The latter figures were crude because the readings obtained were near the lower limit for the method.

EFFECT OF PERIODIC REMOVAL OF NECTAR ON YIELD OF NECTAR SUGAR & STIGMA EXUDATE. As an indirect test of reabsorption of nectar from the corolla tube, nectar was removed from one member of each of 20 pairs of flowers twice daily, and from the remaining flowers at the end of the 3rd day (Controls). No significant difference in yield was found. It was noted, however, that the stigmatic surfaces of flowers from which multiple collections of nectar had been made remained comparatively dry. In a subsequent test it was found that periodic removal of nectar caused a 50 % reduction in the yield of stigma exudate (expt. 1, table I), suggesting that under normal circumstances part of the stigma exudate is derived from nectar which has been reabsorbed from the corolla tube.

EFFECT OF ANTHER EXCISION ON NECTAR SUGAR YIELD. Data from experiments in which the immature anthers were excised are presented in table II.

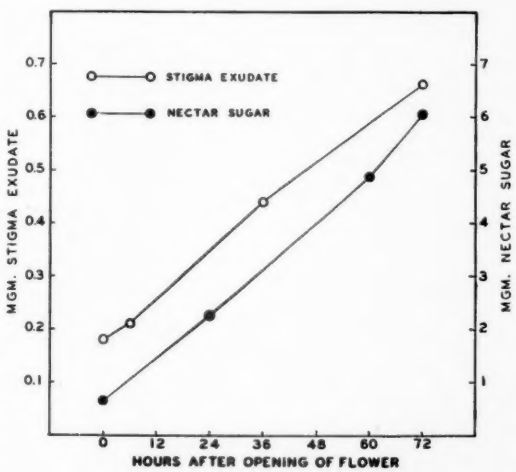

FIG. 2. Comparison of the accumulation of stigma exudate and of nectar in the Streptosolen flower.

TABLE I

EFFECT OF PERIODIC REMOVAL OF NECTAR OR REMOVAL OF IMMATURE ANTHERS ON WEIGHT OF EXUDATE SECRETED BY STIGMAS OF STREPTOSOLEN JAMESONII FLOWERS

EXPT.	NO. OF REPLICATES	TREATMENT	YIELD OF STIGMA EXUDATE, mg	P*
1	13	Control	0.25 ± 0.03**	<0.01
		Nectar removed twice daily	0.12 ± 0.03	
6	22	Control	0.27 ± 0.02	<0.001
		Anthers removed	0.12 ± 0.02	

* Probability of a similar effect by chance
** Standard error of mean

An increase in yield of nectar sugar of 15 or 20% was common, though in experiment 5 the increase was negligible. Two other effects of anther removal always appeared: an accelerated development of the normal red corolla pigmentation and a large reduction in the weight of stigmatic exudate (table I, expt. 6).

INFLUENCE OF STIGMA TREATMENTS ON NECTAR SUGAR YIELD. The results of applying various experimental treatments to the stigma are listed in table III. Removal of the stigma (SR) invariably reduced nectar yield by some 20 to 30% (expts. 7–11). In experiment 8 some stigmas were replaced on the styles over a thin film of lanolin immediately after excision (SRR). Although the stigmas were desiccated after 5 or 6 hours their presence was accompanied by a restoration of the normal nectar sugar yield. The effect of applying pure lanolin to the cut surface of the style was tested in several groups of flowers. With one exception, where a slight increase over the SR group was obtained, lanolin alone did not affect secretion.

In experiment 9 a lanolin paste containing the equivalent of the crude acidic portion of the ether extract of five stigmas (SRE) was placed on the style of each decapitated pistil. Again normal secretion was restored. An equivalent amount of the neutral ether fraction had no effect.

It appeared then that the presence of the stigma was essential for maximal nectar secretion, and that the stigmatic influence was due to the production of an ether-soluble acid. The effect of the acidic extract was simulated by applying 0.5% 3-indoleacetic acid (IAA) in lanolin to the style (expt. 10, treatment SRA). Alpha-naphthaleneacetic acid was equally effective.

CHROMATOGRAPHIC SEPARATION & TESTING OF UNKNOWN SUBSTANCES IN STIGMATIC EXTRACT. The crude acidic fraction was chromatographed in three solvent systems, synthetic IAA being included in each. The R_f values were as follows: in NH_3–H_2O–saturated butanol, first unknown (E_1) 0.66, second unknown (E_2) 0.80, IAA 0.38; in isopropanol–NH_3–H_2O (10:1:1 v/v/v), E_1 0.72, E_2 0.78, IAA 0.26; in *n*-butanol-ethanol-H_2O (4:1:1 v/v/v), E_1 0.62, E_2 0.81, IAA 0.64.

The first unknown gave a pink color product with the Salkowski reagent. The second unknown was either a yellow pigment or was closely associated with a yellow pigment which gave a blue-green color in the presence of ammonia; on drying, the original color returned. Under ultra-violet light the pigment fluoresced white. Sufficient material is not yet available for complete characterization of the unknown substances; obviously neither was IAA.

Each of the unknown substances was incorporated in lanolin at a concentration approximating that used for the crude extract and tested for activity (expt. 11, treatments SRE_1 and SRE_2). Both increased nectar secretion over the SR group, though not to the same extent as the crude acidic extract.

TABLE II

EFFECT OF ANTHER REMOVAL ON NECTAR SUGAR YIELD IN STREPTOSOLEN JAMESONII

EXPT.	NO. OF REPLICATES	TREATMENT	YIELD OF NECTAR SUGAR mg	P*
2	7	Control	3.8 ± 0.23**	<0.01
		Anthers removed	4.9 ± 0.23	
3	23	Control	4.2 ± 0.19	<0.01
		Anthers removed	5.0 ± 0.19	
4	17	Control	4.3 ± 0.18	<0.01
		Anthers removed	5.1 ± 0.18	
5	14	Control	4.1 ± 0.17	>0.05
		Anthers removed	4.5 ± 0.18	

* Probability of a similar effect by chance
** Standard error of mean

Absorption & Movement of C^{14}-Labelled Sucrose in Pistil. Radioautographs showed that C^{14}-sucrose was absorbed and translocated in the pistils of all groups of flowers. An intense exposure of the film in contact with the nectary after 2 days, while development of the remainder of the autograph was still faint, indicated that the nectary was the major site of absorption, although absorption through other parts of the ovary surface in contact with the radioactive sucrose solution cannot be ruled out.

Counts of radioactivity from two experiments are listed in table IV. The data for experiment 12 are incomplete in that activities in the styles were not measured, but are included to show certain variations in the activity patterns. Extreme variability among replicates within treatments indicated that sucrose absorption followed a non-normal distribution; logarithms of counts and geometric means were therefore used in the statistical analysis of activities (20).

In experiment 13 radioactivity in the pistils of mature and senescent flowers amounted to 8 to 9 % of the activity of the solution introduced into the corolla tube. Activity in the pistils of emasculated flowers was significantly less. The differences in total activity between young, and mature or senescent flowers was slightly below the level of statistical significance. On the basis of counts on ovaries and stigmas the young flowers in experiment 12 appeared to absorb much less sucrose than the other groups.

Under the conditions of the experiment, inter-treatment variation in radioactivity in the pistils might have been caused by differences in concentration of C^{14}-sucrose molecules in the solution in the corolla tube, as well as by differences in the absorptive capacity of the nectaries. Nectar was being secreted in all flowers except the senescent, and this would have diluted the C^{14}-sucrose solution. It would be expected from the graph in figure 2 that such dilution would have been similar in extent in young and mature flowers. In emasculated flowers secretion may be increased by as much as 20 %, and this may have accounted for part of the reduction in radioactivity in the pistils of these flowers, though hardly for all of it.

The pattern of distribution of radioactivity within the pistils was variable. In the stigmas of young flowers the proportion of the total activity in the pistil, though not the total count, was consistently higher than in the other groups. In experiment 12 the ratio of activity in the stigma to activity in the ovary was higher in mature than in senescent flowers, whereas in experiment 13 the reverse was true. Although removal of the anthers reduced the total ac-

Table III

Influence of Various Treatments of Stigma on Nectar Sugar Yield in Streptosolen Jamesonii

Expt.	No. of replicates	Treatment	Yield of nectar sugar mg	P*
7	35	Control	3.8 ± 0.11**	<0.001
		Stigma removed	3.0 ± 0.11	
8	18	Control (C)	3.3 ± 0.15	C, SRR vs SR <0.01
		Stigma removed (SR)	2.6 ± 0.15	
		Stigma removed & replaced (SRR)	3.2 ± 0.15	
9	25	Control (C)	5.6 ± 0.17	C, SRE vs SR <0.001
		Stigma removed (SR)	4.3 ± 0.17	
		Stigma removed, crude Stigma extract applied to style (SRE)	5.5 ± 0.17	
10	18	Control (C)	4.1 ± 0.12	C, SRA vs SR <0.001
		Stigma removed (SR)	3.1 ± 0.12	
		Stigma removed, IAA applied to style (SRA)	3.9 ± 0.12	
11	23	Control (C)	5.2 ± 0.15	C vs all others <0.01 SRE_1, SRE_2 vs SRL <0.05
		Stigma removed, lanolin applied to style (SRL)	3.6 ± 0.15	
		Stigma removed, 1st unknown from stigma extract applied to style (SRE_1)	4.1 ± 0.15	
		Stigma removed, 2nd unknown from stigma extract applied to style (SRE_2)	4.2 ± 0.15	

* Probability of a similar effect by chance
** Standard error of mean

TABLE IV

RADIOACTIVITY IN PISTILS OF STREPTOSOLEN JAMESONII FLOWERS IN WHICH NECTAR IN COROLLA TUBE WAS REPLACED WITH A C^{14}-LABELLED SUCROSE SOLUTION

EXPT.	FLOWER GROUP	GEOMETRIC MEANS OF cpm			
		OVARY	STYLE	STIGMA	TOTAL*, **
12	Young (Y)	370		319	
	Mature (M)	7,447		1,355	
	Senescent (S)	4,227		279	
	Anthers removed (AR)	3,493		525	
13	Young (Y)	2,109	2,323	892	6,040
	Mature (M)	12,830	1,239	809	16,050
	Senescent (S)	8,570	5,130	2,113	16,330
	Anthers removed (AR)	2,421	877	762	4,195

* Statistical Significance: Experiment 13 M,S>AR at 5 % level. Activity of C^{14} solution added to corolla tube: experiment 12, 140,800 cpm.; experiment 13, 185,800 cpm.

** As geometric means are used, a mean total activity is not necessarily numerically equal to sum of mean activities of parts.

tivity in the pistils in experiment 13, the counts per minute in the stigma were of the same order as in the control (mature) flowers. As the usual reduction in quantity of stigma exudate was noted in the emasculated flowers, this reduction was evidently not due to any interference with sugar transport within the pistil.

Radioautographs of chromatographed stigma exudate revealed activity in the glucose and fructose components (though not in the sucrose).

About 0.2 % of the activity supplied to the stigmas of newly opened flowers was recovered in the nectar after 24 hours (based on 5 replicates). Activity was found in the sucrose and fructose of radioautographs of chromatographed nectar.

DISCUSSION

The nectary and the secretory tissue of the stigma have several features in common: they are similar anatomically, their periods of activity coincide, and they are both subject to some degree of control by the anthers. Although their respective secretions differ in composition, some interchange of sugar molecules appears to take place between them. Radioactive hexoses derived from C^{14}-labelled sucrose introduced into the corolla tube were found in the stigma exudate, and radioactivity from C^{14}-sucrose applied to the stigmatic surface was recovered in the nectar. The data of experiment 1 (table I) suggest that a large portion of the stigma exudate is normally derived from nectar reabsorbed from the corolla tube.

By analogy with observed rates of absorption of C^{14}-sucrose, it may be inferred that reabsorption of 8 or 9 % of the nectar sucrose present in the corolla tube occurs between the 2nd and 3rd days of secretion. Reabsorption occurs throughout the secretory period. Similarly, sucrose in the stigma exudate may be reabsorbed concurrently with the outward passage of sugars. A common mechanism may serve for outward transport (secretion) and inward transport (absorption). The absorption of sucrose by the nectaries of senescent flowers which had ceased to secrete nectar suggests that secretion may be terminated by a failure in the supply of sugars to the nectary rather than by a failure of the secretory mechanism.

As a normal level of secretion was not attained in the absence of the stigma, production of at least part of the nectar must be under its control. The stigmatic effect is evidently hormonal, residing in the acidic portion of the ether-soluble fraction. Each of two unknown substances separated by paper chromatography from a crude acidic ether extract increased nectar yield when applied to the styles of emasculated flowers. Apparently a specific hormone is not required, as synthetic IAA and NAA produced the same result. Nectar secretion is influenced by auxin level in flowers of *Impatiens holstii*, *Abutilon striatum*, and *Euphorbia pulcherrima* (12) and *Antirrhinum majus* (19). There may be a connection between the stigmatic influence on secretion in Streptosolen and the increase in the absorption and retention of carbohydrates by tomato ovaries following the application of growth regulators to the style (11).

The influence of the anthers was in contrast to that of the stigma in that nectar yields were usually increased by their removal. As anthers are centers of growth hormone production in some species (6, 23) the anther effect may be hormonal. This possibility would be difficult to test directly by an in situ application of a growth substance because of the extreme fragility of the filaments. The increase in nectar with anther removal may be related to the reduction in reabsorption of sucrose through the nectary, and to the polarized transport of sugar in pollinated maize flowers observed by Loomis (8). The exact relationship between the increase in nectar and the reduction in stigma exudate following anther removal is not evident. As secretion of exudate in emasculated

flowers was evidently not limited by the supply to the stigma of sugars reabsorbed from nectar, the effect of anther removal may be exerted at the secretory site in the stigma.

It is evident that in Streptosolen nectar secretion is not an isolated phenomenon, but is related to the metabolism of the anthers and stigma and to the movement of sugar in the flower. It seems likely that maturation of the reproductive organs and secretion are co-ordinated, rather than merely coincidental, events.

Summary

1. The floral nectary and the stigma of *Streptosolen jamesonii* resemble each other with respect to structure and secretory activity. The secretions of the two tissues differ in composition. Data from experiments in which nectar was removed from the corolla tube at frequent intervals, and from experiments with C^{14}-sucrose, indicated that part of the stigmatic exudate is derived from reabsorbed nectar. Conversely, sugar reabsorbed from the stigma exudate may appear in nectar.

2. The quantity of nectar produced is subject to regulation by the anthers and stigma. The quantity of stigma exudate is under the influence of the anthers.

3. Removal of the immature anthers a few hours before secretion commenced usually increased nectar sugar yields by 15 to 20 %. Anther removal consistently reduced the amount of stigmatic exudate.

4. Removal of the stigma caused a reduction in nectar sugar yield of the order of 25 %. Immediate replacement of the stigma, or the application of either the crude acidic fraction of an ether extract of stigmas, IAA, or NAA restored secretion to the normal level. Each of two unidentified substances separated from the crude acidic fraction of the ether extract by paper chromatography gave a partial restoration of normal secretion.

5. Radioactive sucrose, substituted for the nectar sugar in the corolla tube, was absorbed into the ovary (presumably through the nectary) and distributed throughout the pistil. As absorption occurred during the secretory period, reabsorption of part of the sucrose component of nectar must be concurrent with secretion. Ovaries of senescent flowers also absorbed C^{14}-sucrose. The secretory tissue of the stigma, too, is capable of absorbing sucrose.

6. It appears likely that maturation of the reproductive organs and secretion of sugars by the nectary and the stigma are co-ordinated rather than merely coincidental events in this species.

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1. Authorship. List family name of first or sole author before listing initials or given name. Thereafter write names and initials (or given name) of all coauthors in their natural order. Use initials for a man's given name; write out a woman's given name: Reeves, A. C., R. D. Jones, and Helen Smith.

2. Date. The year belongs immediately after the author's name.

H. HYPHENS. Avoid dividing a word at the end of a line. Such division increases the likelihood of a printer's error.

I. UNDERLINING. In general, *do not underscore anything.* When copy is marked for the printer, headings, scientific names, etc., will be underscored in the Editor's office. Exception: *rarely* a word or phrase may be underlined for appropriate emphasis.

J. SPELLING. Draw a box around unusual spelling to indicate that the word has been spelled correctly.

III. PREPARING TABLES.

Set up tables in a form consistent with recent issues of PLANT PHYSIOLOGY. Number tables consecutively in Roman numerals and place the author's name on the back of each page.

A. COPYFITTING. Tables should be preplanned to fit one or two printed columns (3 inches or 6⅛ inches wide, respectively) and not to exceed 8½ inches in height, including heading, body, and footnotes. Tables may not be rotated 90°.

On the average, about 52 typewriter spaces (excluding the new executive typewriter) fill one 3-inch line of type of the size normally used in tables; ten double spaced typewritten lines require 1 inch of the available vertical printed space.

B. NUMERALS. Check tabular data as well as numerical values reported in the text for the proper number of significant figures. Frequently authors report more digits than are significant. Use three dots (*not* a dash) to indicate a blank in a table: . . . For decimals smaller than one, insert a zero before the decimal point: 0.349.

C. FOOTNOTE DESIGNATIONS. Refer to footnotes by the asterisk, double asterisk, triple asterisk, dagger, double dagger, and section, in this sequence as needed: *, **, ***, †, ‡, §. *Place all explanatory material in footnotes* rather than in the heading or columnar headings, both of which should be as *brief as possible* without sacrificing clarity.

D. HEADINGS & FOOTNOTES. Wherever possible, omit unnecessary articles such as "the", "a", in headings and notes. Also avoid bulky prepositional phrases.

E. RULES. Do not use vertical rules. Attractive tables can be achieved by proper spacing without using rulings (which are set by hand, & therefore costly). See current issues for those horizontal rules which are permitted.

IV. ILLUSTRATIONS.

A. PRINTS FOR REVIEW. For manuscript review it is satisfactory to furnish illustrations which have been reproduced by such economical copy methods as Verifax and blue print. Thus prints which will be used for publication need not be sent through the mail repeatedly. *Do not send original drawings or photos* at any time.

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1. Kinds of Illustrations. Figures are of two kinds, line drawings and halftones. Line drawings are just that—line drawings usually done with India ink on white paper. Halftones, in contrast to line drawings, are not just black and white, but they also are shaded from black to white. Because halftones are photographed through a screen some detail is lost in reproduction.

2. Special Treatment of Photographs. For illustrations in which it is especially important to bring out all the detail present in the original photograph special processing will be undertaken. Should your paper contain electron-micrographs, radioautographs, or other material whose reproduction should be deemed critical, call the editor's attention to the need for special processing.

3. Good Illustrations. Plan in advance for clear, legible illustrations. Line drawings should be intensely black on white; halftones should have good contrast and be in sharp focus throughout. No glossy should contain blemishes. Do not use paper clips on photos; grooves, gouges, and creases show when reproduced.

Generally the appearance of a graph is improved if the top and right sides are boxed in by lines of the same weight as the abscissa and ordinate.

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Figures are reproduced the same size (x 1) or they are reduced. (Enlarging magnifies defects.) Illustrations are fitted either into 3 inch or 6⅛ inch column widths. Although

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5. Grouping. One large illustration is cheaper to reproduce than three to six small ones, so *as far as possible group all figures.* Mount similar figures close together. Place halftone and line drawings in separate groups.

Limit the area of each photograph to that which is necessary to show pertinent details. Trim off irrelevant portions and minimize blank space.

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7. Identification. Place the author's name on the back of each figure and legend. Place the figure number on the back of each figure. Identification insures against misplacement.

V. SUBMITTING MANUSCRIPTS.

Send the original copy of the manuscript, the first carbon, and two copies of all illustrative material to the Editor, Dr. Allan H. Brown: Department of Botany: University of Minnesota: Minneapolis 14, Minn. Retain a carbon copy of your manuscript and original photographs or drawings to insure against loss.

PLANT PHYSIOLOGY does not accept a manuscript which has not been submitted directly by an author. If your manuscript is submitted by someone other than one of the authors, send a letter to this office stating that you wish to publish that particular manuscript in PLANT PHYSIOLOGY. Your manuscript cannot be processed until this letter is received. This precaution protects you as well as the Journal.

VI. PROCESSING MANUSCRIPT.

A. REVIEW. The manuscript usually will be sent to two reviewers familiar with that field of research covered by the paper. Each reviewer evaluates the manuscript, suggests improvements, and recommends accepting or rejecting the paper. Usually two months suffice for review and decision. When there is marked disagreement between reviewers, the Editor may appoint a referee (often a member of the Editorial Board) to evaluate the manuscript in light of the reviews; the referee recommends either for or against acceptance.

B. REVISION. Manuscripts returned to the author for revision should be sent back to the Editor within two months; otherwise the paper will be published under the date of receipt of the revised manuscript and not the original date.

Retype each page on which there are extensive changes.

C. GALLEY PROOF. This will be sent to the person designated on page 1 above the title. (See II D 1).

1. Returning Proofs. Return only one set of galley proofs to Editor. Keep any other set for reference. Be certain to notify Editor promptly of change of address, so galleys can be sent to the correct address.

2. Charges for Errors. You will be charged at cost for all changes or corrections in galley and page proof which are your fault. You will not be billed for errors which are not your fault. Here are two ways to minimize charges:

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E. ERRATA. If necessary, an errata will be published for each volume. Authors therefore are requested to call the attention of the Editor to any significant errors in their published manuscripts.